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L-amino acid oxidase with cytotoxic activity from Aplysia punctata

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L-amino acid oxidase with cytotoxic activity from Aplysia punctata

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20 Jan. 2003

L-amino acid oxidase with cytotoxic activity from Aplysia punctata

Description

The present invention relates to a cytotoxic polypeptide which is an L-amino acid oxidase isolated from the ink of the sea hare Aplysia punctata.

The sea hare Aplysia produces a pink-coloured ink, which has cytotoxic activity towards several eukaryotic cell lines. WO97/16457 discloses a partial sequence from an Aplysia protein, which allegedly has anti-tumor activity. Cyplasin L (558 aa, NCBI accession number 11967690) and cyplasin S (421 aa, 11967688; Petzelt and Werner, 2001, Cell Biology International, 25(2):A23) both include parts of sequences disclosed in WO 97/16457. Cyplasin S exhibits 95% sequence identity to cyplasin L. Cyplasin L is produced in the nidamental gland but neither in the ink gland (including the mantle region) nor in the opaline gland of Aplysia punctata. Thus, it is concluded that cyplasin is not a component of Aplysia ink and is not responsible for the cytotoxic activity of the Aplysia ink. A detailed description of Aplysia anatomy and a dissection guide can be found in the internet in Richard Fox, Invertebrate anatomy (1994, http://www.science.lander.edu/rsfox/).

The overall aim in tumor therapy is the selective eradication of transformed cells without harming healthy cells. Several glycoproteins isolated from sea hares (Aplysia species) have attracted attention because of their anti-tumor activity, e.g. aplysianin A from Aplysia kurodai, or cyplasins. The underlying mechanism for such activity has however not been elucidated so far. Recombinant intracellular cyplasins seem to be non-toxic, whereas the extracellular cyplasin is cytotoxic (Petzelt et al., Neoplasia, 4:49-59, 2002).

WO 02/31144 discloses a further cytotoxic factor isolated from the ink of Aplysia punctata. Fragments of the amino acid sequence of the factor are disclosed. No data were presented demonstrating that this factor has any oxidase function or has any properties related to an oxidase.

At least two main phenotypes of cell death are described: apoptosis, a genetically fixed physiological form of cell death, is accompanied by shrinkage, membrane blebbing, nuclear fragmentation, and final disintegration into so-called apoptotic bodies. In contrast, necrosis is a pathological process characterized by membrane disruption and cell swelling. Cell death induced by reactive oxygen and nitrogen species (ROS/NOS) might lead to apoptosis and necrosis but also to other forms of cell death, which cannot be clearly assigned to one of these main forms of cell death.

The cytotoxic factors derived from the sea hares so far have several disadvantages which might hamper its application. The biological function and the nature of the cytotoxic activity, which are prerequisites for the development of a lead compound, are not known so far. Aplysianin A contains a dinucleotide binding fold and the so-called "GG motif" which are found in many flavoproteins. The GG motif has also been described in cyplasins (Petzelt et al., supra). Based on this knowledge, the factors can be applied in its entirety only, because the domains relevant for proper function and cellular receptors are unknown. The administration of an entire non-self protein to an animal or a human might cause severe immunologic complications.

The dinucleotide binding fold and the GG motif are found e.g. within the N-terminal domain of FAD containing enzymes (e.g. reductases, dehydrogenases, hydroxylases, peroxidases, and oxidases). FAD containing enzymes can be classified into five groups GR1, GR2, FR, PCMH, and PO according to the sequences of their FAD binding domains

and additional conserved sequence motifs (Dym and Eisenberg, Protein Science, 10:1712-1728, 2001). The consensus sequence of GR1 and GR2 is GxGxxG. The GG motif RhGGRhxxT/S is commonly found in oxidases, e.g. L-amino acid oxidases, monoamino oxidases, polyamine oxidases, and putrescine oxidases, wherein x describes any amino acid, and h describes a hydrophobic amino acid.

L-amino acid oxidases catalyse the formation of H_2O_2, ammonia, and an alpha keto acid from an amino acid in the presence of oxygen and H_2O (Geyer et al, 2001, Eur. J. Biochem. 268, 4044-4053). An L-lysine alpha oxidase (EC 1.4.3.14) for instance can be obtained from the fungus Trichoderma spec. (Kusakabe et al., J. Biol. Chem. 10:976-981, 1980) which shows antimetastatic effects (Umanskii et al., Biull Eksp Biol Med. 109:458-9, 1990, Khaduev et al., Biull Eksp Biol Med. 112:419-22, 1991). The Trichoderma L-lysine oxidase is a dimer with a molecular weight of 112-119 kDa. A further L-lysine oxidase obtained from the fish Chub mackerel is a dimer and has a molecular weight of 135 kDa (Jung et al., J. Immunol. 165:1491-1497, 2000) and induces apoptosis. Apoxin is an L-leucin oxidase from the rattlesnake (Crotalus atrox) venom which induces apoptosis in tumor cells and vascular endothelial cells in vitro (Torii et al., J. Biol. Chem. 272:9539-9542, 1997). A cytotoxic L-lysine alpha oxidase is described in the art which penetrates into Jurkat cells and there activates oxidative deamination of L-lysine and correspondingly the peroxide formation. Conjugates of the enzyme with monoclonal antibodies against the CD5 receptor cannot penetrate into the cells and are assumed to produce toxic H_2O_2 outside the cells. The conjugates have a reduced cytotoxic effect, although the effect of conjugation upon enzymatic activity is negligible (Zhukova et al., Vopr Med Khim 2001, 47:588-592). Another L-lysine oxidase obtained from the snail Achatina fulica and producing H_2O_2 is found to have an antimicrobial effect. This oxidase might be useful as an agent against pathogenic bacteria (Ehare et al., 2002, FEBS Letters, 531:509-512).

Most known alpha amino acid oxidases which produce H_2O_2 possess a broad substrate specificity. The L-lysine alpha oxidase from Trichoderma viride (EC 1.4.3.14, Kusakabe et al., supra) is specific for lysine, but also oxidizes L-ornithine, L-phenylalanine, L-tyrosine, L-arginine, and L-histidine to a lesser extent. The L-lysine oxidase of Chub mackerel (EMBL, AJ400781; Jung et al., supra) is specific for lysine and in addition transforms arginine, histidin, leucine, methionine, phenylalanine, and ornithine (specifity 40 fold reduced). Even if these enzymes could be cytotoxic due to their ability to produce H_2O_2, a therapeutic use is hampered because substrates of these enzymes are available in the body fluid in amounts sufficient to release H_2O_2 everywhere in the body. Under these conditions, possible negative side effects of H_2O_2 are difficult to eliminate.

Thus, the problem underlying the present invention is the provision of a means for selective generation of H_2O_2 in target tissues, e.g. in tumor tissues with less toxic side effects upon normal cells. The solution is a cytotoxic polypeptide which can be isolated from the ink of the sea hare *Aplysia punctata* and which is a specific L-lysine and/or L-arginine oxidase producing H_2O_2 or a fragment or derivative of said polypeptide. The activity of the enzyme can be modulated be administration of substrate. The enzyme provides a lead structure, and it can be used for target identification.

A first aspect of the present invention is a purified polypeptide which exhibits cytotoxic activity on tumor cells and which comprises the amino acid sequence shown in SEQ ID NO:2, 4, or 6, or a cytotoxic fragment thereof. These sequences are derived from a cytotoxic 60 kDa protein purified from crude ink of Aplysia punctate via anion exchange chromatography and gel filtration (see examples 1 and 4). Thus, the polypeptide or the fragment is termed APIT (*Aplysia punctata* ink toxin).

The purity of the fractions can be determined by SDS-PAGE and silver staining.

The cytotoxic activity of APIT or the diluted crude ink can be measured by the reduction of the metabolic activity of eukaryotic cells. A person skilled in the art knows suitable methods and cell lines. For example, the metabolic activity of Jurkat T cells can be measured by the addition of WST-1, which is a tetrazolium salt converted by cellular enzymes of viable cells, e.g. by the mitochondrial dehydrogenase, to a dark red formazan. Therefore, the amount of formazan correlates with cell vitality. Formazan can be determined photometrically at 450 nm. Further, dead eukaryotic cells killed by APIT or the diluted crude ink can be counted by adding propidium iodide (PI) at 1 μg/ml in PBS and subsequent flow cytometer analysis. PI is a DNA binding dye which is taken up by dead cells with permeable membranes.

The cytotoxic activity of APIT is reduced by at least 70% after 10 min incubation at 60°C. At 70°C, the activity is almost absent, whereas 0°C to 50°C have no effect upon the activity. APIT shows a loss of activity with decrease of pH, with complete inactivation after 10 min pre-incubation at pH 3. After 30 min treatment with 6 M urea, the activity of APIT is almost unaffected. At 8M urea, the activity is reduced by about 50% (example 3).

Tumor cells treated with APIT displays a morphology which is neither typical for apoptosis nor for necrosis but rather is typical for oxidative damage induced cell death. Shrunken nuclei and lack of cell swelling are apoptotic, and early membrane permeabilization is a necrotic characteristic (example 2). The phenotype induced by APIT could be reproduced in Jurkat cells by treatment of the cells with concentrations of H_2O_2 >200 μM, indicating that H_2O_2 is the active compound in APIT cytotoxic effect. H_2O_2 concentrations <100 μM induced apoptosis in Jurkat cells.

By depriving possible substrates which can be converted into H_2O_2 from the culture medium of the tumor cells, it can be demonstrated that no further toxic effect of APIT upon tumor cells is present. Deprivation of L-lysine and L-arginine from the medium prevents cell death completely. In a detailed analysis of the enzymatic activity of APIT, media containing single amino acids (20 L-amino acids, D-lysine) confirmed that L-lysine and/or L-arginine is converted into H_2O_2 and the respective alpha keto acid to the same extent, whereas no conversion could be measured with any other of the remaining 18 L-amino acids and D-lysine (example 7). The production of H_2O_2 is independent of the presence of cells, however, the presence of cells reduces the amount of free H_2O_2, which might be due to detoxification of the medium by the cells. Catalase (a H_2O_2 hydrolyzing enzyme) prevents tumor cell death induced by purified APIT and by crude ink as well, confirming the conclusion that H_2O_2 is responsible for the ink mediated killing of tumor cells (example 6).

In summary, the data demonstrate that the polypeptide of SEQ ID NO:2, 4, or 6 (APIT) is an oxidase which is capable to produce H_2O_2. Particularly, the polypeptide is an alpha amino acid oxidase. More particularly, the polypeptide specifically converts L-lysine and/or L-arginine in the presence of O_2 and H_2O into an alpha keto acid, ammonia, and H_2O_2. Thus, the polypeptide is preferably an L-lysine and/or L-arginine oxidase.

A characteristic feature of the active fractions containing APIT purified from crude ink were two absorption maxima at 390 nm and 470 nm, a hallmark of flavoproteins. A flavine nucleoside, particularly FAD is required as a co-factor for the anti-tumor and oxidase activity of APIT as removal of FAD inactivated APIT (example 5).

Analysis of the sequences SEQ ID NO:2, 4, and 6 revealed that APIT comprises a sequence similar to known dinucleotide binding folds which

are characteristic for flavoproteins (Fig. 4c). The GG-motif (consensus sequence RhGGRhxT/S) is found adjacent to the dinucleotide binding fold.

A further aspect of the present invention is a polypeptide comprising a fragment of the polypeptides of the sequences of SEQ ID NO:2, 4, or 6 which can be used as a lead structure for drug development. APIT can be digested by a protease without loss of activity. Digestion leaves the substrate specifity unaltered. Thus, the fragment exhibiting cytotoxic activity is an L-lysine and/or L-arginine oxidase. Preferably, proteinase K is used which is a relative unspecific protease resulting in small fragments. Other proteases which can be selected among specific or unspecific proteases known by a person skilled in the art can be used instead of proteinase K. The cytotoxic proteinase resistant domain of APIT is of particular importance for the development of a non-immunogenic, fully active small compound.

Further preferred fragments comprise partial amino acid sequences of APIT which are obtained by peptide mass fingerprinting, ESI/MS, and Edman degradation:

DG(I/V)CRNRRQ
DSGLDIAVFEYSDR, VFEYSDR
LFXYQLPNTPDVNLEI (X=T in SEQ ID NO:2, 4 and 6)
VISELGLTPK
GDVPYDLSPEEK
VILAXPVYALN (X=M in SEQ ID NO:2, 4 and 6)
ATQAYAAVRPIPASK
VFMTFDQP
SDALFFQMYD (FFQ is FSQ in SEQ ID NO:2, 4 and 6)
SEASGDYILIASYADGLK
NQGEDIPGSDPQYNQVTEPLK (PQY is PGY in SEQ ID NO:2, 4 and 6)

While not wishing to be bound by theory, the FAD group which is tightly bound to the amino acid chain, e.g. by a covalent bond, might cover possible protease cleavage sites. Thus, protease treatment results in a fragment comprising the active centre of the enzyme, including the prosthetic group FAD. This conclusion is confirmed by the finding that native APIT cannot be cleaved by trypsin, but trypsin can digest denaturated APIT.

Thus, an especially preferred fragment of APIT which is an oxidase exhibiting cytotoxic activity is a sequence comprising the dinucleotide binding fold and the GG motif corresponding to amino acid residues No. 39 to 77 in SEQ ID NO:2. This sequence is identical to the sequence of amino acid residues No. 38 to 76 in SEQ ID NO:4 and No. 21 to 59 in SEQ ID NO:6. More preferably, the fragment has an L-lysine and/or an L-arginine oxidase activity.

Further, the fragment can comprise a stretch of additional amino acid residues which may be selected from SEQ ID NO:2 or 4 from the sequences adjacent to the residues No. 39 to 77 in SEQ ID NO:2 or No. 38 to 76 in SEQ ID NO:4. Preferably, 1-20 additional amino acid can be present at the N-terminus and/or the C-terminus. More preferably, 1-10 additional amino acid can be present at the N-terminus and/or the C-terminus. Most preferably, 1-5 additional amino acid can be present.

A further aspect are polypeptides which are homologous to the polypeptides of SEQ ID NO:2, 4, or 6, or to fragments thereof, which have an identity of at least 70%, preferably at least 80%, more preferably at least 90%, or most preferably at least 95%. SEQ ID NO: 2, 4, or 6 describe natural variations of APIT by replacements of single amino acids not affecting its function. In further 11 clones, four mutations were found within the sequence comprising the dinucleotide binding fold and the GG motif (Pos. 39 to 77 in SEQ ID NO:2, see example 4). Taking into account

that a fragment obtained by proteolytic digestion is still active as a L-lysine and/or L-arginine oxidase, it can be expected that further modifications of the sequence, e.g. by amino acid substitutions, deletions and/or insertions will not substantially affect the function of APIT. A modified sequence exhibits an identity of preferably at least 70%, more preferably at least 80% and most preferably at least 90% to a reference sequence, e.g. SEQ ID NO:2. Preferably, the sequence of Pos. 39 to 77 in SEQ ID NO:2 has a higher degree of identity to the reference sequence than the total amino acid sequence, e.g. preferably at least 33 of 39 amino acid residues (at least about 85%), more preferably 35 of 39 residues (at least about 90%), and most preferably 37 of 39 residues (at least about 95%).

A still further aspect is a polypeptide of the present invention as described above which is a recombinant polypeptide. The recombinant polypeptide is characterized as being manufactured in a heterologous, i.e. non-Aplysia host cell, e.g. in a bacterial cell such as E. coli or Bacillus, in a yeast cell such as saccharomyces cerevisiae, in an insect cell or in a mammalian cell. The recombinant polypeptide has preferably an oxidase, or, more preferably, an L-lysine and/or an L-arginine oxidase activity. Expression of the polypeptide can be done by standard expression systems known by a person skilled in the art. For proper enzymatic function, the prosthetic group FAD may have to be introduced into the polypeptide.

The protein of the invention or a fragment thereof may be in the form of a fusion protein, i.e. fused to heterologous peptide or polypeptide sequences. Preferably fusion proteins are genetic fusions, wherein the nucleic acid sequence encoding a protein or a protein fragment as described above is fused to a nucleic acid sequence encoding a heterologous peptide or polypeptide sequence. The heterologous peptide or polypeptide sequence may be selected from signal sequences, which provide desired processing and/or transport in a host cell. The signal sequence is preferably located at the N- and/or C-terminus of the APIT sequence. Further examples of

heterologous sequences are domains which assist expression in host cells and/or purification from cellular extracts or culture media. Still further examples of heterologous sequences are targeting sequences which may direct the APIT polypeptide to a desired target site, e.g. in an organism. Suitable targeting sequences may be e.g. single chain antibodies, which may be directed against tumor specific antigens or proteinaceous ligand sequences, which may be directed against tumor specific receptors.

A further aspect of the present invention is a nucleic acid coding for the polypeptide as described above. The total mRNA of the mantle gland, the nidamental gland, the digestive gland, and the opaline gland can be prepared by standard methods. The mRNA can be reverse transcribed using the tagged oligo dT oligonucleotide (Oligo 1, Fig. 4b). The tag is a random sequence not expected to be present within Aplysia mRNA to be reverse transcribed. PCR can be performed using the degenerated primer (Oligo 2) derived from the APIT peptide VFEYSDR and the specific primer (Oligo 3) directed against the tag sequence of the oligo dT primer Oligo 1. The amplified sequence can be cloned into a standard vector and can be sequenced by standard techniques. By this strategy, the 3′ terminal sequence of the APIT gene can be obtained. The 5′ terminal sequence can be obtained by the RACE strategy. The mRNA from selected tissues (see above) is reverse transcribed using an oligonucleotide derived from the known 3′ terminal sequence (e.g. Oligo 4, or Oligo 6) and can be treated with a terminal transferase in the presence of CTP, resulting in a 3′-poly-C-sequence (at the minus strand). PCR can be performed using a tagged primer against the poly-C-sequence (Oligo 5) and a specific primer, e.g. Oligo 4, or Oligo 6. The amplified product can be cloned and sequenced by standard techniques. Finally, for obtaining full-length cDNA clones, specific primers, e.g. Oligo 8 and Oligo 9 can be used. By this strategy, three different clones were obtained and sequenced. The nucleotide sequences are described in SEQ. ID. No.1, 3, and 5 which are

identical to 97% (1560 of 1608) of the nucleotides. 42 of 48 mutations are silent mutations which have no effect upon the amino acid sequence.

By this strategy, further clones of APIT can be obtained which might have a differing sequence. Since more than ten sequences of APIT are known, specific or degenerated primers may be selected from these sequences, and new clones can be obtained by a single PCR of reverse transcribed mRNA.

Thus, the nucleic acid encoding a polypeptide as specified above preferably comprises

(a) a nucleotide sequence as shown in SEQ ID NO:1, 3, or 5, or at least the polypeptide coding portion thereof, or the complement thereof, or

(b) a nucleotide sequence corresponding to the sequence of (a) within the scope of degeneracy of the genetic code, or the complement thereof, or

(c) a nucleotide sequence hybridizing under stringent condition with the sequence of (a) and/or (b), or

(d) a nucleotide sequence which is homologous to the sequences of (a) and/or (b).

The nucleic acid may be a single stranded or double stranded nucleic acid (DNA or RNA). The nucleic acid is obtainable from natural sources e.g. from *Aplysia* by extraction of RNA, construction of cDNA libraries and screening of the library using degenerated oligonucleotides which were deduced from the peptide sequences described above. The nucleic acid is further obtainable by RT-PCR using RNA extracted from *Aplysia* and oligo-dT-primers or degenerated primers. On the other hand, the nucleic acid is obtainable by chemical synthesis.

Hybridization under stringent conditions preferably means that after washing for 1 h with 1 x SSC and 0.1% SDS at 55°C, preferably at 62°C and more preferably at 68°C, particularly after washing for 1 h with 0.2 x SSC and 0.1% SDS at 55°C, preferably at 62°C and more preferably at 68°C, a hybridization signal is detected.

The degree of identitiy of the nucleic acid is at least 70%, preferably at least 80%, more preferably at least 90%, and most preferably at least 95% to a reference sequence, e.g. SEQ ID NO: 1, 3 or 5.

Further, the nucleic acid encoding a cytotoxic polypeptide can comprise a partial sequence of the nucleotide sequence as disclosed in SEQ ID NO:1, 3, or 5. Preferably, the partial sequence is selected from nucleotide No. 115 to 231 in SEQ ID NO:1, or nucleotide No. 112 to 228 in SEQ ID NO:3, or nucleic acid residue No. 61 to 177 in SEQ ID NO:5, or the partial sequence codes for at least one of the eleven fragments of APIT obtained by peptide mass fingerprinting, ESI/MS, and Edman degradation. Further, the partial sequence can comprise a stretch of additional nucleotides selected from the sequences adjacent to the sequence selected from SEQ ID NO:1, 3, or 5. Preferably, 1-60 additional nucleotides can be present at the 5' and/or the 3'-terminus. More preferably, 1-30 additional nucleotides can be present at the 5' and/or the 3'-terminus. Most preferably, 1-10 additional nucleotides can be present at the 5' and/or the 3'-terminus.

Furthermore, the nucleic acid may encode a fusion polypeptide as described above.

In a preferred embodiment of the invention the nucleic acid is operatively linked to an expression control sequence, e.g. a sequence which is capable of directing expression in a suitable host cell, e.g. a prokaryotic or eukaryotic host cell. The expression control sequence usually comprises a promoter and optionally operator or enhancer sequences which enable a

transcription of the nucleic acid operatively linked thereto. Furthermore, the expression control sequence may contain a translation signal, e.g. a ribosome binding sequence.

The nucleic acid of the present invention may be a recombinant vector which contains in addition usual vector sequences such as an origin of replication, a selection marker gene and/or a cloning site. Examples of suitable vectors such as plasmids, phages or viral vectors are known to the skilied person and are described e.g. in Sambrook et al., Molecular Cloning, A Laboratory Manual (2nd ed. 1998), Cold Spring Harbor, Laboratory Press.

A further aspect of the present invention is a recombinant cell transformed or transfected with a nucleic acid as described above. The recombinant cell may be a prokaryotic cell, e.g. a gram-negative prokaryotic cell such as E. coli or an eukaryotic cell, e.g. an insect cell or a vertebrate cell such as a mammalian cell. Techniques for transforming or transfecting host cells with nucleic acids are known to the skilled person and e.g. described in Sambrook et al., supra.

Still a further subject matter of the present invention is an antibody directed against the polypeptide as described above. The antibody may inhibit the cytotoxic activity of the polypeptide. The antibody may be a polyclonal or monoclonal antibody or a recombinant antibody, e.g. a chimeric antibody, a humanized antibody or a single chain antibody. Furthermore, the antibody may be an antibody fragment containing the antigen-binding site of the antibody, e.g. a Fab fragment. The antibody may be obtained by immunizing suitable experimental animals with an *Aplysia* polypeptide as described above or a partial fragment thereof or a peptide antigen optionally coupled to a suitable macromolecular carrier according to known protocols, e.g. by techniques which are described in Borrebaeck, Carl A.K. (Ed.), Antibody engineering (1992), or Clark, M.

(Ed.), Protein engineering of antibody molecules for prophylactic and therapeutic applications in man (1993). By techniques for producing hybridoma cell lines according to Köhler and Milstein monoclonal antibodies may be obtained.

Methods for introducing a prosthetic group into a polypeptide are known in the art. Preferably, the FAD is introduced by a method comprising surface display of the polypeptide on a prokaryotic host, comprising the steps:

(a) providing a prokaryotic host cell transformed with a nucleic acid fusion operatively linked with an expression control sequence, said nucleic acid fusion comprising sequences necessary for displaying the protein on the outer membrane, and

(b) culturing the host cell under condition wherein the nucleic acid fusion is expressed and the expression product comprising the recombinant polypeptide is displayed on the surface of the host cell, and

(c) contacting the recombinant polypeptide with FAD under conditions wherein FAD combines with the recombinant polypeptide and a functional recombinant polypeptide containing the prosthetic group is formed.

The nucleic acid fusion may be formed using a nucleic acid sequence as described above and further sequences necessary for surface display. Details describing the prokaryotic host cells, the sequences necessary for surface display of the polypeptide, culture conditions, and the conditions under which the recombinant polypeptide is contacted with FAD are described in WO 02/070645, which is included by reference herein.

A further aspect of the present invention relates to diagnostic or therapeutic applications in humans or animals. The polypeptide, and/or a nucleic acid, and/or a recombinant cell, and/or an effector, e.g. an inhibitor

or activator of the polypeptide as described above can be used in such applications. The polypeptide as described above is able to selectively kill tumor cells. For example, T and B leukemia cell lines, a chronic myeloid leukemia cell line (K562), cells from an orphan and aggressive osteosarcoma (Ewings tumor: RDES, A673), a small cell lung cancer cell line (GLC4, GLC4/ADR), cervix cancer (Chang), and acute monocytic leukemia (THP-1) show an $IC_{50} \leq 10$ ng/ml APIT.

Resistance to apoptosis as well as multi drug resistance (MDR) represent severe problems in cancer therapy. It is therefore of particular interest that the polypeptide of the present invention kills apoptosis resistant cell lines as well as MDR cancer cell lines to the same extent as their non resistant counter parts. Over-expression of apoptosis inhibitors of the Bcl-2 family in cancer cell lines does not protect from APIT mediated cell death, confirming that APIT induces cell death in an apoptosis independent way. The MDR cell line GLC4/ADR possess almost the same sensitivity to APIT (IC_{50} 10 ng/ml) as the parental cancer line GLC4 does (IC_{50} 9 ng/ml).

Thus, the diagnostic or therapeutic application preferably relates to a method for diagnosis or treatment of hyperproliferative diseases, e.g. cancer. More preferably, the method is a method for diagnosis or treatment of lung cancer, breast cancer, prostate cancer, colon cancer, cervix cancer, uterus cancer, larynx cancer, stomach cancer, liver cancer, Ewings sarkoma, acute lymphoid leukemia, acute and chronic myeloid leukemia, apoptosis resistent leukemia, and/or MDR lung cancer. Moreover other tumor types can also be treated with the polypeptide, like pancreas cancer, gastric cancer, kidney cancer, gliomas, melanomas, chronic lymphoid leukemia, and/or lymphoma. Since all cancer cell lines tested (in total 24) were effectively killed by APIT, the polypeptide can be used for the treatment of solid tumors and leukemias in general including apoptosis resistant and multi drug resistant cancer forms.

A further aspect of the present invention is a pharmaceutical composition comprising the polypeptide of the present invention as described above, in a pharmaceutically effective amount and optionally together with suitable diluents and carriers or kit containing the composition together with other active ingredients, e.g. modulators of the polypeptide or other cytostatic or cytotoxic agents. The composition can be administered locally or systemically by any suitable means, e.g. orally, nasally or by injection (i.v., i.p., s.c., or i.m.) to a subject in need thereof. The components of a kit, which consists of at least two different compositions may be administered together or separately, e.g. at different times and/or by different routes.

From many studies it is known that tumor cells have an increased rate of metabolism compared to normal cells. A result of this high metabolic rate is a high concentration of reactive oxigen species (ROS, comprising H_2O_2) which originate from oxidative phosphorylation reactions by the electron transport chain of the mitochondria. As a consequence ROS detoxification reactions are increased in tumor cells, and interference with detoxification has a selective toxic effect on the tumor cells but not on normal cells. Likewise, increasing the concentration of H_2O_2 by administering the polypeptide of the invention in a predetermined amount may overcome the detoxification reactions and kill the tumor cells. The level of extra H_2O_2 produced by exogenous APIT does not affect normal cells because of their higher tolerance for additional H_2O_2. An administration of the polypeptide in a varying amount, e.g. a gradually changing, e.g. increasing amount leads to the production of a defined amount of H_2O_2 could thus be used for a selective killing of cancer cells.

The pharmaceutical composition or kit as described above can comprise a further component which is a substance capable of modulating the cytotoxic acitivity of the polypeptide, in a pharmaceutically effective amount and optionally together with suitable diluents, and carriers. In FCS (100%) at 37°C and 5% CO_2 which reflect *in vivo* conditions, or in a

medium containing 10% FCS (typical *in vitro* conditions) devoid of L-lysine and L-arginine, the activity of APIT (20 ng/ml) can be dose-dependently increased by the addition of L-lysine in a final concentration of 2 – 50 μg/ml. Thus, the high specifity of APIT for L-lysine (and L-arginine) allows for modulating the enzymatic activity of APIT and thus its cytotoxic activity by providing an additional substrate *in vivo* or *in vitro*. The substance capable of modulating the cytotoxic activity of the polypeptide can be L-lysine, L-arginine, a derivative or metabolic precursor of L-lysine, or L-arginine, or a mixture thereof. A derivative is a compound which is an APIT substrate. A metabolic precursor is a compound, which can be metabolized to a compound, which is an APIT substrate. Further, the modulator may be selected from flavine nucleosides, particularly FAD, since the presence of a flavine nucleoside prosthetic group leads to a great increase in APIT activity.

The pharmaceutical composition may comprise the polypeptide and at least one modulating substance as a mixture. Preferably, the modulating substances are provided in a kit consisting of separate preparations. More preferable, the polypeptide is provided for administration before the modulating substances.

During the passage through body fluids before reaching the tumor tissue, the cytotoxic activity of the polypeptide would be undesired, due to the toxic properties of H_2O_2. Thus, the composition may further comprise an inhibitor of the polypeptide. The inhibitor could have a short half-life time in the body fluid. A preferred inhibitor of the polypeptide is an antibody against the polypeptide (see above).

Further the polypeptide can be coupled with a substance and/or a particle which targets the polypeptide to the tumor tissue.

Further components of the pharmaceutical composition can be a nucleic acid coding for the polypeptide as described above, and/or a recombinant vector or cell containing the nucleic acid.

A further aspect of the present invention is a substance modified by interaction with APIT (termed target substance of APIT). A direct interaction is a contact of APIT with this substance. In an indirect interaction, the effect upon the substance includes at least one mediator substance, e.g a substance formed by APIT, or a receptor interacting with APIT and the components of the related transduction cascade.

As described above, a mediator of APIT acting on cellular polypeptides is H_2O_2. Thus, preferred target substances of APIT comprise cellular polypeptides, which can be modified by H_2O_2. A major modification identified in 2-DE SDS gel patterns of cells treated with APIT was a shift of peroxiredoxin I (Prx I, Swiss-Prot No. Q06830, Genbank identifier No. 548453), which was also detected in cells treated with H_2O_2. Prx I belongs to a class of peroxidases which are involved in the detoxification of ROS. Although the nature of the modification of Prx is not known, PrxI can be used as a marker for APIT anti-tumor activity.

WO 02/31144 discloses proteins modified by H_2O_2 which are targets of APIT: thioredoxin peroxidase 2 (Swiss Prot No. Q06830, Genbank identifier 548453), 60S ribosomal protein P0 (12654583), Hsp-60 (N-term) (14603309), stathmin (5031851), Rho GDI 2 (P52566, 1707893), 60S ribosomal protein P0(4506667), RNA binding regulatory subunit (O14805,12720028), hnRNP C1/C2 (4758544), hnRNP C1/C2 (4758544), proteasome subunit beta type 1 (P20618, 130853), pre-mRNA cleavage factor Im (5901926), proteasome subunit alpha type 7 (O14818, 12643540), U2 small nuclear ribonucleo-protein A´ (P09661, 134094), GAP SH3 binding protein (5031703), DNA replication licensing factor MCM4 (P33991, 1705520), thioredoxin peroxidase 1 (P32119, 2507169),

40S ribosomal protein S21 (P35265, 464710), 40S ribosomal protein S12 (P25398, 133742), phosphoglycerate mutase 1 (P18669, 130348), HCC-1 protein (13940310), HnRNP A2/B1 (4504447/14043072), IMP dehydrogenase 2 (P12268, 124419), hnRNP A/B (14724990).

Further targets of APIT identified by 2 DE gel electrophoresis, in-gel tryptic digestion, peptide mass fingerprinting by MALDI-MS, and identification of the proteins are summarized in Table 3.

Still a further target of APIT is a nucleic acid. The target nucleic acid can be a DNA or an RNA, which is a mRNA. The transcription of the mRNA is up- or downregulated in the presence of APIT and/or H_2O_2. Preferably, the transcription is changed by a factor of at least 2, and more preferably, by a factor of at least 4.

By a microarray of specific 60mer oligonucleotides representing about 8500 human genes, about 70 mRNAs were identified which are targets of APIT. The information about the mRNAs are summarized in Table 4. Each mRNA is referenced by a "unigene cluster" which represents a number of nucleotide sequences belonging to the same gene or to closely related genes. Details of the nomenclature and the nucleotide sequences of the unigene clusters are public available under http://www.ncbi.nlm.nih.gov/ (Homepage of the National Center for Biotechnology Information).

For most of the unigene clusters of Table 4, the gene and/or the protein is known. It is a general principle that modulation of the transcription of a messenger RNA influences the amount of protein expressed. Thus, the proteins coded by the sequences of the unigene clusters of Table 4 are also targets of APIT, because APIT may influence their expression. The sequences of the proteins and of the nucleic acids coding for these proteins are referenced by the genbank identifier, accession number and/or

version number (see Table 4). The sequences are public available under http://www.ncbi.nlm.nih.gov/.

The target substance of the present invention (see Table 3 and 4), which is identified by one of the methods as described above, may be used for the development of new pharmaceutical agents, e.g. by known high-throughput screening procedures which may be cellular screening procedures or molecular based screening procedures. These pharmaceutical agents may act upon cellular receptors and/or components of the signal transduction pathways activated or inhibited by APIT.

Degenerative diseases like Alzheimer's and Parkinson's disease are characterised by excessive ROS production of the affected tissue. Drugs which either activate H_2O_2 detoxification or inhibit H_2O_2 production may be used for therapy of degenerative diseases like Alzheimer's or Parkinson's disease. Fast growing tumor cells produce more ROS and thus require an efficient H_2O_2 detoxification system. Drugs which either activate H_2O_2 production or which interfere with H_2O_2 detoxification may be used for therapy of proliferative diseases like tumors. Since e.g. thioredoxin peroxidases 1 and 2 have been shown to be overexpressed in cells at risk for diseases related to ROS toxicity including degenerative diseases like Alzheimer's and Parkinson's disease, and have been shown to be overexpressed in tumor cells (Butterfield et al., 1999, *Antioxidants & Redox Signalling,* 1, 385-402), the targets of Table 3 and 4 might be important targets for the development of drugs for treatment of degenerative diseases like Alzheimer's and Parkinson's disease and of proliferative diseases like tumors.

NK-cells have been shown to protect against malignant cells in chronic myelogenous leukemia (CML), but their number and inducibility is reduced during the progression of the disease. This reduction and dysfunction is due to the production of H_2O_2 by CML-cells (Mellqvist, Blood 2000, 96,

1961-1968). NK-cells encountering H_2O_2 are inhibited in their lytic activity, are made resistant to IL-2 activation and undergo apoptosis/necrosis. Any therapy providing CML-patients with ROS-hyposensitive NK-cells therefore would be of great benefit. The targets described above could be used to modulate the H_2O_2 sensitivity of NK-cells or to inhibit the H_2O_2 production of malignant cells, e.g. CML-cells.

Arteriosclerosis with its progression to heart disease, stroke and peripheral vascular disease continues to be the leading cause of death in all western civilisations. Enhanced ROS-production (via endothelial NADPH-oxidase) is required and sufficient to generate the pathologic phenotype (Meyer, FEBS Letters 2000, 472, 1-4). Therefore, targets mediating the effect of H_2O_2 are useful to develop new drugs for treatment of arteriosclerosis and the associated diseases like heart disease, stroke and other vascular diseases. These targets are suitable to detoxify H_2O_2 and/or to block the H_2O_2 induced signalling pathways.

Target compounds, e.g. peptides, polypeptides or low-molecular weight organic compounds, which are capable of modulating the effect of H_2O_2 may be identified in a screening system comprising the use of the APIT polypeptide as described above. Particularly, a modulation of the APIT activity, i.e. L-amino oxidase activity, may be determined.

Thus the present invention further relates to a pharmaceutical composition comprising as an active agent at least one of the target substances as described above.

The invention is explained in more detail by the following figures, tables and examples.

Figure 1

A, Anion exchange chromatography. Filtrated and concentrated ink was loaded onto a Source Q15 column. Proteins were eluted by a linear gradient from 0 to 800 mM NaCl, fractions were collected every minute (2 ml/min). Absorption was measured at 280 nm. Horizontal bar indicates active fractions.

B, Gelfiltration. Active fractions from the Source Q15 were pooled and concentrated and applied to a Superose 12 HR 10/30 column. Proteins were eluted with 100 mM potassium phosphate buffer (pH 7.2). Fractions were collected every minute (0.5 ml/min). Horizontal bar indicates active fractions.

Figure 2

A, Phenotype of APIT-induced cell death. Jurkat cells were cultured for 7 hours in the presence (APIT) or absence (medium) of APIT (30 ng/ml) and phase contrast images were recorded.

B, Lack of apoptotic DNA fragmentation in ink-treated cells. Jurkat cells were incubated in medium (control) or treated with cycloheximide (chx; 10 μg/ml) or ink (ink, 1/500 diluted) for 2, 4 and 6 h. Isolated DNA was visualized on a 1,6% agarose gel by ethidium bromide staining.

C, APIT mediated loss of metabolic activity. APIT (10 ng/ml) and the tetrazolium-salt WST-1 were added simultaneously to Jurkat cells and turnover of WST-1 was measured photometrically. White circles: medium control; black circles: APIT-treated samples; mean absorbance of 8 replicates ± SD.

D, Cell death induced by ink. Jurkat cells were treated with ink (1/500 diluted) and propidium iodide (PI) uptake was measured as indicator for dead cells.

Figure 3

A, Heat sensitivity of ink. Dialysed ink was incubated for 10 min at the indicated temperatures and enzymatic activity was measured as H_2O_2-production (mean of triplicates ± SD). Blank: medium control.

B, pH-sensitivity of APIT. APIT (60 ng) was incubated for 10 min at 25°C in 0,1 M potassium phosphate at indicated pH values. Enzymatic activity was measured as H_2O_2-production (mean of triplicates ± SD).

C, Sensitivity to increasing amounts of urea: Dialyzed ink (black bars, 1/500 diluted) and as positive control 0,625 mM *α*-keto isocaproic acid (open bars) were treated with indicated concentrations of urea for 30 min at 25°C. Enzymatic activity (15 min, 25°C) was measured as *α*-keto acid formation via MBTH.

Figure 4

A, N-terminal and internal peptide sequences of the APIT protein.

B, List of oligonucleotides used for cloning of the APIT gene.

C, Nucleotide sequence of the APIT cDNA and the derived amino acid sequence. The dinucleotide binding fold (VAVVGAGPGGANSAYMLRDSG LDIAVFE) and the GG-motif (RVGGRLFT) are indicated by boxes. Consensus amino acid residues are indicated by bold letters. The N-terminal sequence of mature APIT (dashed line) and of internal peptides (solid line) derived by Edman degradation and mass finger prints are

indicated. Sequence variations of the three clones are indicated by small boxes.

D, Variation of the N-terminus of APIT in 11 further clones.

Figure 5

A, Anion exchange chromatography of purified APIT. Proteins were eluted by a linear gradient from 0 to 800 mM NaCl and fractions were collected every minute. Absorption was measured at 280 nm (AU: Absorption unit).

B, Fractions 24, 27 and 29 were separated by SDS-PAGE and tested for metabolic activity by WST-1 assay. High activity (+; ++) correlated with the presence of a prominent 60 kDa band (fractions 24 and 29). Activity is given as the dilution leading to > 85% reduction of the metabolic activity of Jurkat cells (+/- = 1:900; + = 1:2700; ++ = 1:8100).

C, Absorption spectra of fractions 24 (black line), 27 (dashed line) and 29 (dotted line).

Figure 6

A, APIT induced H_2O_2 production in medium in the absence of cells. APIT (260 ng/ml) was incubated in medium in the presence (open bar) or absence (black bar) of Jurkat cells ($5x10^5$/ml). After 1 h of incubation at 37°C supernatants were alkylated with N-ethylmaleimide and H_2O_2 was measured (mean values of 3 independent experiments +/- SD).

B, Catalase inhibits ink induced cell death. Jurkat T-cells were incubated for 8 h with ink in the presence (black bars) or absence (white bars) of catalase. Cytotoxicity was measured as PI uptake (mean of triplicates ± SD).

C, Catalase protects from APIT induced loss of metabolic activity. Metabolic activity of Jurkat cells was measured after incubation with APIT (20 ng/ml) or anti-CD95 for 3h in the presence (black bars) or absence (white bars) of catalase. (mean of 5 replicates ± SD).

D, Phenotype of APIT induced cell death is mediated by hydrogen peroxide. Jurkat cells were cultured for 7 hours in the presence (APIT) or absence (medium) of APIT (60 ng/ml) or H_2O_2 (500 μM) and were analyzed by phase contrast microscopy. Catalase was added in combination with APIT to neutralize H_2O_2 (APIT+CAT).

Figure 7

A, Enzymatic activity of APIT in the presence of different medium supplements. APIT (200 ng/ml) was incubated for 60 min at RT with RPMI +/- 10% FCS or KRG supplemented with different medium ingredients and H_2O_2 production was measured. (EAA = essential amino acids, NEAA = non essential amino acids, concentrations see Table 1).

B, Substrate specificity of APIT and ink. The enzymatic reaction of dialysed ink (open bars) with different L-amino acids in potassium phosphate buffer was measured as H_2O_2-production. 50 μM H_2O_2 and amino acid free medium (control) were used as control. Aliquots of dialyzed ink were digested with trypsin (hatched bars) or proteinase K (black bars) at 37°C for 2h prior to testing the substrate specificity. Arg = L-arginine, 1mM; Lys = L-lysine, 1mM; EAA = essential amino acids, 1mM; NEAA = non essential amino acids, 1mM.

C, APIT induced cell death depends on the presence of L-lysine or L-arginine. Jurkat cells were incubated with APIT (20 ng/ml) for 6 h in the presence (white bars) or absence of L-lysine and L-arginine (black bars). Cytotoxicity was measured as PI uptake (mean of triplicates ± SD).

D, APIT induced loss of metabolic activity depends on the presence of L-lysine or L-arginine. Jurkat cells were incubated with APIT (20 ng/ml) or anti-CD95 (150 ng/ml) in the presence (open bars) or absence (black bars) of L-lysine or L-arginine and metabolic activity was measured (mean of 5 replicates ± SD).

E, APIT transforms L-lysine into an α-keto acid. APIT was incubated with L-lysine and the formation of α-keto acid was measured photometrically by its reaction with MBTH.

F, Michaelis-Menten kinetic of APIT activity with L-lysine. K_m value for L-lysine was determined as H_2O_2 production.

G, Proposed reaction mechanism of L-amino acid oxidases according to Macheroux et al. (2001 Eur. J. Biochem. 268:1679-1686). Encircled are compounds which we demonstrated to participate in the reaction catalyzed by APIT.

Table 1

Composition and concentrations of mixtures of essential and non-essential amino acids as well as single amino acids used in Fig. 7A.

Table 2

APIT kills different kinds of tumor cells. Different tumor cell lines (50,000 cells in 100 μl) were incubated for 14 h in the presence of increasing amounts of APIT. Metabolic activity of the cells was measured via turnover of WST. The IC_{50} values reflect the APIT concentration at which the metabolic activity is decreased to 50%. (* stands for $IC_{50} \geq 20$ ng/ml at the given cell concentration of 50,000/100 μl.)

Table 3

List of proteins which were changed in their expression or modified after treatment with APIT (upregulation (+), downregulation (-), or modification (m) in column "effect"). The proteins are referenced by the genbank identifier and/or accession number and/or version number.

Table 4

List of genes (referenced by unigene cluster number) and gene products (proteins) which were modulated in their expression more than 2 fold after incubation with APIT for two hours. The proteins are referenced by the genbank identifier and/or accession number. Transcription rates are indicated as increase (+, 2 to ≤ 4 times; + +, 4 to 6 times) or decrease (-, 2 to ≤ 4 times; - -, 4 to 6 times).

Example 1: Purification of APIT

Aplysia punctata were gained from the Station Biologique Roscoff, Bretagne, France. Crude ink was prepared by gentle squeezing the sea hares in sterile seawater. Insoluble particles were removed by ultracentrifugation (82,000g, 30 min, 4°C) and supernatants were stored at −70°C.

APIT was purified from crude ink via anion exchange chromatography and gelfiltration. The thawed ink was filtered through Whatman filter No. 4 under slight vacuum and subsequently through a 5 μm and 0.45 μm syringe filter. The filtrate was concentrated by using Ultrafree-15 Units (Millipore, exclusion weight 30 kDa) followed by three washing steps with 20 mM Tris HCl (pH 8.2). After centrifugation at 10.000 g for 5 min the supernatant of the concentrate (20 – 60 fold) was applied to a Source Q15 column ((10mm, length 40 mm) equilibrated with 20 mM Tris HCl, pH 8.2. Proteins were eluted by a linear gradient from 0 to 800 mM NaCl over 50 ml at a flow rate of 2 ml/min (Fig. 1A). The purity of the fractions was determined by SDS-PAGE and subsequent rapid silver staining. APIT

appears as a band at 60 kDa. Cytolytic activity was measured as APIT-induced reduction of the metabolic activity of Jurkat cells via turnover of WST (see example 2). Enzymatic activity was determined as described in example 3. Fractions which show high purity and cytotoxic respectively enzymatic activity (Fig. 1A; fraction 42 to 48) were pooled, concentrated and loaded onto a Superose 12 HR 10/30 column (Pharmacia). Proteins were eluted with 100 mM potassium phosphate buffer (pH 7.2) at a flow rate of 0.5 ml/min The first peak represents the active APIT (Fig. 2B; fraction 11 to 14).

Example 2: Phenotype of APIT-induced cell death

The purple fluid of Aplysia punctata contains a cytolytic activity which induces rapid and extensive death of Jurkat T cells in culture. APIT induces cell death of tumor cells which resembles neither apoptosis nor necrosis. In order to classify the APIT-induced cell death we looked for common features of apoptosis and necrosis.

Jurkat T cells were harvested in the log phase, centrifuged and adjusted to a density of 5×10^5/ml with fresh medium (RPMI supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin). Cells were cultured with APIT, cycloheximide as a positive control or medium at 37 °C, 5% CO_2 and 100% humidity for the indicated times. Fragmented DNA of apoptotic cells was analyzed according to Herrmann et al. (1994, Nucleic Acid Research 22: 5506-5507). Cell vitality was determined as metabolic activity via the turnover of WST-1 (ROCHE, Mannheim) to red formazan by the mitochondrial dehydrogenase of viable cells. Absorbance of the cell suspension was measured photometrically at 450 nm (690 nm reference). Toxicity was measured by quantifying propidium iodide uptake (1 μg/ml in PBS) by Flow Cytometry.

Morphologically, tumor cells treated with ink or APIT did not exhibit typical morphological apoptotic or necrotic signs of cell death (Fig. 2A), and neither blebbing nor swollen cells were detected when cells were treated with a lethal dose of ink. Cells did not form clusters anymore, cytoplasm became translucent and nuclei prominent (Fig. 2A). The intracellular movements of plasma and organelles stopped, detachment and formation of vacuoles were observed when adherent cells were incubated with APIT (data not shown). Consistent with the absence of apoptosis, fragmented DNA or nuclei were not detected in ink-treated tumor cells (Fig. 2 B); moreover, caspases were not activated (data not shown). Metabolic activity of tumor cells was blocked as early as 30 min after exposure to ink or APIT (Fig. 2C). Ink-treated tumor cells rapidly took up propidium iodide (PI) indicating plasma membrane permeabilization and cell death (Fig. 2D).

Example 3: Stability of APIT

APIT was further characterized by its sensitivity to heat, low pH and high concentrations of urea.

For determination of its heat sensitivity native ink was dialyzed against PBS at 4°C for several days to separate chromopeptides. Dialysed ink was incubated for 10 min at the indicated temperatures, and activity was measured immediately as enzymatic production of H_2O_2. This assay is based on the finding that APIT transforms L-lysine to H_2O_2 and α-keto acid. The production of H_2O_2 was determined via the turnover of ABTS (2,2-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) to a green formazan in the presence of H_2O_2 by horseradish peroxidase. Heat-treated ink was incubated with L-lysine (1 mM) in 100 μl 100 mM potassium phosphate buffer, pH 7.2 for 10 min at 25°C. The reaction was stopped by adding 1 μl of 10 M phosphoric acid. To 25 μl of this solution 1 mM ABTS and 1 Unit horseradish peroxidase was added in 225 μl 100 mM potassium

phosphate buffer, pH 5.0. Absorption was measured photometrically at 405 nm (reference 690 nm).

Purified APIT was challenged to different pH-values by adding a mixture of monobasic and dibasic potassium phosphate and phoshphoric acid rendering the desired pH. After a 10 min incubation pH of samples was adjusted to pH 7.2 by adding appropriate amounts of dibasic phosphate. Afterwards enzymatic activity was measured as H_2O_2-production as described above.

The activity of APIT after treatment with urea was measured via the production of α-keto acid, which was quantified photometrically by its reaction with the hydrazone MBTH (3-methyl-2-benzothiazolone hydrazone hydrochloride) as described by Soda (1968). Dialyzed ink was incubated with urea at indicated concentrations for 30 min. Subsequently the remaining enzymatic activity was measured without removing urea for 15 min at 25°C. As control, defined amounts of α-keto isocaproic acid (Sigma; K-0629) were treated equally.

APIT was characterized by its heat sensitivity and was found to exhibit a high and constant activity after pre-incubation for 10 min at 0°C to 50°C. Activity was clearly reduced at 60°C and absent at temperatures of 70°C or higher (Fig. 3A). APIT also shows a loss of activity with decreasing pH, with complete inactivation after a 10 min pre-incubation at pH 3 or lower (Fig. 3B). An outstanding feature of APIT is its resistance to urea (Fig. 3C). After 30 min treatment with 6 M urea, the activity of APIT was almost unaffected. At 8 M urea, the activity was reduced by about 50%.

Example 4: Sequencing and cloning of APIT

In order to clone the cDNA of APIT N-terminal and internal peptide sequences were identified by PMF (peptide mass fingerprint), ESI/MS and

Edman degradation (Fig. 4A). A suitable internal peptide sequence was used to design a degenerated primer for PCR (Fig. 4A, underlined sequence) with reverse transcribed mRNA, prepared from Aplysia punctata tissues. Subsequent 5'-RACE yielded the full length cDNA which was cloned and analyzed.

Amino acid sequencing by peptide mass fingerprint (PMF), ESI/MS and Edman degradation. Purified APIT was separated by SDS PAGE and 2 DE gel electrophoresis (Thiede et al., 2001, J. Biol. Chem. 276: 26044-26050). The N-terminus of APIT was identified from a single band/spot of a PVDF blot by Edmann degradation. For the identification of internal peptide sequences a single band/spot was punched from the gel, digested with trypsin and dissolved in aqueous trifluoroacetic acid (Thiede et al., 2001, J. Biol. Chem. 276:26044-26050). Tryptic peptides were separated using a Smart-HPLC system with a column of 2.1 mm inner diameter and 10 cm length (μRPC C2/C18 SC 2.1/10, Smart System, Pharmacia Biotech, Freiburg, Germany) and an acetonitrile gradient in 0.1% (v/v) trifluoroacetic acid at a flow rate of 100 μl/min at room temperature. The peptide fractions were dried, dissolved in 6 μl 0.3% (v/v) aqueous trifluoroacetic acid/acetonitrile (2:1) and analyzed by MALDI-MS. The mass spectra were recorded by using a time-of-flight delayed extraction MALDI mass spectrometer (Voyager-Elite, Perseptive Biosystems, Framingham, MA, USA) as previously described (Thiede et al., 2001, J. Biol. Chem. 276:26044-26050). Briefly, fifty mg/ml 2,5-dihydroxybenzoic acid in 0.3% (v/v) aqueous trifluoroacetic acid/acetonitrile (2:1) was used as matrix and 0.3 μl of the sample and 0.3 μl of the matrix were mixed and applied to a gold-plated sample holder and introduced into the mass spectrometer after drying. The spectra were obtained in the reflectron mode by summing 50-150 laser shots. For N-terminal sequencing peptide fractions containing single masses were loaded onto a Biobrene-coated glass fiber filter, transferred to a PVDF membrane and excised. Sequencing was performed using a Procise sequencer (Applied Biosystems, Weiterstadt, Germany).

Cloning of the APIT gene. In order to dissect mantle gland, nidamental gland, digestive gland and opaline gland some animals were relaxized by injection of 5 – 10 ml sterile $MgCl_2$ solution (380 mM). Isolated tissues were frozen immediately in liquid nitrogen. Total RNA was prepared from these tissues using the „peq gold TRIfast" reagent (Peqlab). mRNA was reverse transcribed using the tagged oligo dT oligonucleotide 5'-tcc taa cgt agg tct aga cct gtt gca $t_{(18)}$-3' (Fig. 4B, oligo 1) and the Superscript II polymerase (LIFE) at 42°C. In order to amplify a fragment of the APIT gene the degenerated primer 5'-tc gtg ttc gar tac tci gay cg-3' derived from the APIT peptide VFEYSDR (Fig. 4B, oligo 2) and the specific primer 5'- ctg tag gtc tag acc tgt tgc a-3' (Fig. 4B, oligo 3) directed against the tag sequence of the oligo dT-primer was used. PCR was performed with the „expand long template" system (ROCHE, Mannheim) at 68°C and the product was cloned into the pCMV-vector (Stratgene) and sequenced. The 5' terminal cDNA of APIT was cloned using the 5' RACE System (LIFE) according to the manufacturers instructions. Primers 5'-ccg tgt aga tct cac tgc cat a-3' (Fig. 4B, oligo 4) or 5'-ccg ttg agt tgt aga cct-3 (Fig. 4B, oligo 6) were combined with the primers 5'-ggc cac gcg tcg act agt acg ggi igg gii ggg iig-3' (Fig. 4B, oligo 5) or 5'-aatt ggc cac gcg tcg act agt ac-3' (Fig. 4B, oligo 7) to yield a product which was cloned into the pCDNA3-vector (Invitrogen) and sequenced. Finally, full length APIT cDNA was obtained by amplifying the APIT using the specific primers 5' – aa ttc tcg tct gct gtg ctt ctc ct (Fig. 4B, oligo 8) and 5' – gac tta gag gaa gta gtc gtt ga (Fig. 4B, oligo 9) and cloned into the pGEX-4T3 Vector (Amersham). DNA from 3 clones of transfected E.coli was prepared and sequenced.

The identity of the isolated gene was confirmed by comparing the computed translational product (Fig. 4C) with the amino acid sequences of the tryptic peptides (Fig. 4A) and the peptide mass fingerprint. It consisted of 1608 bp coding for a protein of 535 amino acids (Fig. 4C) with the predicted mass of 60,167 dalton and a pI of 4.59. The N-terminal 18 amino acids of APIT comprised a putative secretion signal sequence which

was absent from the mature protein, most likely due to posttranslational modification during secretion. Furthermore, APIT exhibited homology to FAD-binding oxidoreductases with a conserved dinucleotide binding fold around amino acids 39 to 66 followed by a so-called GG-motif typical for certain oxidases like LAAO, MAO (Fig. 4C) (Dailey et al., 1998, J.Biol. Chem. 273:13658-13662; Vallon et al., 2000, Proteins 38:95-114; Macheroux et al., 2001 Eur. J. Biochem. 268:1679-1686). The highest degree of homology existed to the Cyplasin from *A. punctata*, the Aplysianin from *A. kurodai* and the mucus-toxin of the giant African snail *Achatina fulica*.

Comparing the 3 derived DNA-sequences we often found differences in the third position of coding triplets which nevertheless only seldom produced changes in the amino acid sequence of APIT (Fig. 4C).

By the method described above, further 11 clones were isolated from Aplysia punctata which have a homology to the sequences described in Fig. 4 of at least 95%. Several mutations of the amino acid sequence were found in the domain comprising the dinucleotide binding fold and the GG motif, which probably have no effect upon the function (Fig. 4D). In Pos. 22 of SEQ ID NO:2, C is replaced by S in two clones. In Pos. 52, A is replaced by T in one clone. In Pos. 60, L is replaced by Q in 7 clones. In Pos. 69, D is replaced by H in one clone. In Pos. 77, T is replaced by S in one clone.

Example 5: FAD association

The toxic and enzymatic activity of APIT is due to the presence of an attached FAD.

In order to purify the tumor lytic activity, ink from *A. punctata* was subjected to different purification protocols and afterwards each fraction

was tested for its toxic activity (see example 1). Activity always correlated with the presence of a protein of approximately 60 kDa (Fig. 5 A and B). Moreover, APIT was found to contain carbohydrate residues using the DIG Glycan/Protein double labeling method (Roche; data not shown). Furthermore, all spectra of the highly active fractions exhibited a double peak at 390/470 nm (Fig. 5C) which is characteristic for protein bound flavines (Massey et al., 2000, Biochem Soc. Trans. 28:283-96). Heating of APIT for 10 min to 60°C, which is accompanied by a substantial loss of activity also results in loss of detectable FAD-absorption, as is the case with lowering the pH to inactivating values around pH 3. Heating and pH-challenge of APIT was performed as described in example 3 (data not shown).

Consistently, APIT contained the conserved dinucleotide binding fold involved in pyrophosphate binding (Wierenga et al., 1986, J. Mol. Biol., 187:101-107) which is found in many flavoproteins (Fig. 4B; example 4). Moreover, in APIT like in many oxidases a so-called GG-motif is found adjacent to the dinucleotide binding fold (Dailey et al., 1998, J. Biol. Chem. 273:13658-13662, Vallon et al., 2000, Proteins, 38:95-114). Based on the structure of the dinucleotide binding fold and conserved sequence motifs, FAD containing proteins are ordered into 4 families (Dym et al., 2001, Protein Sci. 10:1712-28). According to this classification and based on homology APIT belongs to the Glutathione reductase 2 family (GR2) (Dym et al., 2001, Protein Sci. 10:1712-28). The data show that FAD is a necessary prosthetic group for toxic and enzymatic activity of APIT.

Example 6: Cell-death is mediated via H_2O_2

Proteome analysis revealed that thioredoxin peroxidase II is involved in the APIT mediated tumor cell death. Thioredoxinperoxidase II is involved in detoxification of reactive oxygen species (ROS) by reducing hydrogen

peroxides as well as other peroxides. We therefore tested whether H_2O_2 is produced during APIT incubation and found that H_2O_2 is the mediator of APIT-induced cell death. Scavenging this toxic compound by catalase results in survival of APIT treated cells.

H_2O_2 production was measured after incubation of APIT in medium alone and in cell suspension as described in example 3. Toxicity was measured by quantifying propidium iodide uptake (1 μg/ml in PBS) by Flow Cytometry. Cell vitality was determined as metabolic activity via the turnover of WST-1 (ROCHE, Mannheim) to red formazan by the mitochondrial dehydrogenase of viable cells. Absorbance of the cell suspension was measured photometrically at 450 nm (690 nm reference).

As shown in Fig. 6A, APIT induced the production of H_2O_2 in the presence (167 μM) as well as in absence of cells (280 μM). This strongly argues for an enzymatic activity of APIT which transforms medium ingredients under the production of hydrogen peroxide. In the presence of cells the measured H_2O_2 amount is somewhat lower which might be explained by cellular consumption and degradation of H_2O_2. In the absence of APIT H_2O_2 was not detectable. To investigate whether the APIT-induced cell death is mediated by H_2O_2, cells were treated with APIT in the presence of the H_2O_2 degrading enzyme catalase and then stained with PI. Catalase completely abolished the ink-induced increase of PI stained cells (Fig. 6B). Degradation of H_2O_2 by catalase also inhibited the rapid break-down of metabolic activity induced by APIT (Fig. 6C) but, as expected, was ineffective in blocking CD95 (Fas/Apo-1)-induced cell death in the same assay (Fig. 6C). In the presence of catalase APIT no longer induced morphological changes of tumor cells as judged by microscopic investigation (Fig. 6D). The highly efficient inhibition by catalase in particular suggested that no other substance than H_2O_2 elicits the toxic effect observed in APIT-treated samples. Consistently, H_2O_2 induced the phenotype typical for APIT-treated cells (Fig. 6D). Furthermore, proteome analyses revealed changes in H_2O_2

treated cells which were characteristic of APIT-treated cells. These data together clearly demonstrated that the cytotoxic activity depended on the H_2O_2 producing enzymatic activity of APIT.

Example 7: APIT is a L-lysine/L-arginine a-oxidase. Enzymatic activity is a prerequisite for toxicity

APIT produced H_2O_2 in RPMI medium in the abence of cells. In order to idenitify the substrates in cell culture medium which are converted to H_2O_2 by APIT, we prepared different media with defined amino acid composition by supplementing HEPES buffered modified Krebs Ringer medium (KRG: 25 mM HEPES pH 7.4, 125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 5 mM $NaHCO_3$, 6 mM glucose, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$) with 10% FCS, 2 mM glutamine, essential and non-essential amino acids (Invitrogen), or single essential amino acids in concentrations equivalent to RPMI medium (Invitrogen). Media were adjusted to pH 7.4 and filter sterilized. After incubation of these media with purified APIT the enzymatic activity was measured as H_2O_2 production via turnover of ABTS (2,2-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) to a green formazan in the presence of H_2O_2 and horseradish peroxidase (Fig. 7A and Table 1).

In a next step we checked whether the substrate specificity could be impaired by digest of APIT. For proteolytic digest aliquots of dialysed ink were treated for 2 h with proteinase K (0,05 mg/ml final) in PBS at 37°C. Reaction was stopped by adding aprotinin (1 µg/ml final) or PEFA ([4-(2-aminoethyl)-benzolsulfonyl fluoride-hydrochloride]-hydrochloride; 0,25 mg/ml final), and digest was checked on a 15% SDS-PAGE. After incubation of digested ink with different amino acid compositions in potassium phosphate buffer the enzymatic activity was measured as H_2O_2 production (Fig. 7B).

In order to test whether withdrawal of L-lysine and L-arginine results in rescue of APIT-treated cells we incubated Jurkat cells in medium lacking L-lysine and L-arginine. Control cells were cultured in a medium containing L-lysine(HCl (40 mg/l) and L-arginine(HCl (240 mg/l). Toxicity was measured by quantifying propidium iodide uptake (1 μg/ml in PBS) by Flow Cytometry (Fig. 7C).

Cell vitality was determined as metabolic activity via the turnover of WST-1 (ROCHE, Mannheim) to red formazan by the mitochondrial dehydrogenase of viable cells. Absorbance of the cell suspension was measured photometrically at 450 nm (690 nm reference). As control tumor cells were killed by anti-CD95 treatment (Fig. 7D).

α-Keto acids were quantified photometrically by their reaction with the hydrazone MBTH (3-methyl-2-benzothiazolone hydrazone hydrochloride) as described (Soda et al., 1968, Anal. Biochem. 25:228-235) (Fig. 7E).

The K_m value for L-lysine was determined as H_2O_2 production and calculated according to Michaelis Menten with the GraphPad Prism 3.0 software (GraphPad Software, San Diego California USA) using non linear regression (Fig. 7F).

Surprisingly, from all amino acids tested only L-lysine and L-arginine served as substrates for APIT to produce hydrogen peroxide (Fig. 7A). Moreover, the restricted substrate specificity was even maintained when APIT was digested with protease K suggesting that the protease resistant fragment of APIT contains both, the active domain and the domain which determines the substrate specificity (Fig. 7B). These data were confirmed by functional analyses which showed that APIT was unable to induce cell death (Fig. 7C) or reduce metabolic activity (Fig. 7D) in tumor cells incubated in medium lacking L-lysine and L-arginine, indicating that the enzymatic activity of APIT is the prerequisite for its toxicity. L-lysine and L-arginine deprivation

had no influence on the metabolic activity of tumor cells under the experimental conditions (Fig. 7D). Activation of CD95(Fas/Apo-1) efficiently impaired cell vitality irrespective of the presence of L-lysine or L-arginine (Fig. 7D), demonstrating that cell death can be induced under L-lysine and L-arginine limited conditions.

As shown in the reaction scheme in figure 7G, α-keto derivatives are produced by amino acid oxidases and these could indeed be demonstrated when L-lysine was used as substrate for APIT (Fig. 7E). These results suggested that APIT catalyses the formation of H_2O_2 by the reaction outlined in figure 7G. Kinetic studies analyzed according to Michaelis-Menten revealed a K_m of 0.182 mM for L-lysine (Fig. 7F).

By adding L-lysine (2-50 μg/ml) to tumor cells which are cultured with APIT (20 ng/ml) in medium depleted of L-lysine and L-arginine or in pure FCS, the metabolic activity of the tumor cells can be reduced down to 16% respectively 50% of the control cells without additional L-lysine. This shows that the tumorolytic effect of APIT can be manipulated by changing the amount of available substrate which is of significance for *in vivo* studies and/or for application of APIT in pharmaceutical compositions and/or methods for treatment of cancer.

Example 8: Sensitivity of different tumor cell lines to APIT induced cell death.

Tumor cells were harvested in the log phase. Triplicates of each 50,000 cells were cultured in a flat bottomed 96-well-plate in 100 μl medium with increasing concentrations of APIT. After 14 hours the metabolic activity of the cells was determined by addition of 10 μl WST-1 per well (ROCHE, Mannheim). The yellow tetrazolium salt is cleaved to red formazan by cellular enzymes of viable cells. The metabolic activity correlates with cell

vitality and was quantified by measuring the absorbance of the dye solution with a spectrophotometer at 450 nm (reference 650 nm).

APIT is able to kill different tumor cells. T and B cell leukemia cell lines (Jurkat neo, CEM neo, SKW neo), a chronic myelogenous leukemia cell line (K562), and cells from an orphan and aggressive osteosarcoma (Ewings tumor: RDES, A673) showed the highest sensitivity to the APIT induced cell death ($IC_{50} \leq 5.6$ ng/ml), followed by cells derived from small cell lunger cancer (GLC4, GLC4/ADR), cervix cancer (Chang) and acute monocytic leukemia (THP-1) ($IC_{50} \leq 10$ ng/ml). Most of the adherent growing cells of solid tumors (breast cancer: MCF-7, SK-BR-3; prostate cancer: PC3, DU-145; colon cancer: HT-29; cervix cancer: HeLa; uterus cancer: Hec-1-B; larynx cancer HEp-2; stomach cancer: AGS; liver cancer: Hep G2) and the monocyte leukemia cell line MonoMac 6 are less sensitive at the indicated cell concentration ($IC_{50} \leq 20$ ng/ml), but become more sensitive when lower cell concentrations were used (IC_{50} 5 - 10 ng/ml).

Resistance to apoptosis as well as multi drug resistance (MDR) represent severe problems in cancer therapy. It is therefore of particular interest that APIT kills apoptosis resistant cell lines as well as MDR cancer cell lines equally efficient as their non resistant counter parts (Tab. 2): Over-expression of apoptosis inhibitors of the Bcl-2 family in acute lymphoblastic leukemia cell lines (CEM Bcl-X_L, Jurkat Bcl-2) as well as in B cell leukemia (SKW Bcl-2) (Tab. 2; 4th row) does not protect from APIT mediated cell death and results in IC_{50} values of ≤ 6 ng/ml, similar to the non-transfected parental cell lines, confirming that APIT induce cell death in an apoptosis independent way. The MDR cell line GLC4/ADR (Tab. 2, 5th row) was generated by selection with doxorubicin (Zijlstra et al.,1987; Cancer Res. 47:1780-1784). Its multifactorial MDR is caused by over-expression of MRP-1 and a decreased activity of the DNA topoisomerase II. GLC4/ADR cells possess almost the same sensitivity to APIT (IC_{50} 10 ng/ml) as the parental line GLC4 does (IC_{50} 9 ng/ml).

Example 9: Proteome analysis: change in protein expression pattern in Jurkat T cells after treatment with APIT

Treatment with APIT. Jurkat T cells (5×10^5 /ml) were incubated with APIT (20 ng/ml) for 8 h at 37°C in 5.0% CO2 in the presence of 1 μg/ml cycloheximide. Controls were performed without APIT.

Total cell lysate. The Jurkat T cells were solubilized in 5 volumes of a buffer containing 9 M urea, 25 mM Tris/HCl, pH 7.1, 50 mM KCl, 3 mM EDTA, 70 mM DTT, 2.9 mM benzamidine, 2.1 μM leupeptin, 0.1 μM pepstatin, 1 mM PMSF, and 2% carrier ampholytes (Servalyte pH 2-4, Serva, Heidelberg, Germany). After 30 minutes of gentle stirring at room temperature, the samples were centrifuged at 100000 g (Ultracentrifuge Optima TLX, Beckman, München, Germany) for 30 minutes with a TLA120.2 rotor, which were kept at room temperature before centrifugation. The clear supernatant was frozen at -70°C.

Proteomics. The methods of preparing 2-DE gels, staining with Coomassie Blue G-250, staining with silver nitrate, in-gel tryptic digestion, peptide mass fingerprinting by MALDI-MS, and identification of the proteins are described in Jungblut et al., Molecular Microbiology, 2000, 36, 710-725.

Identification was performed using the peptide mass fingerprinting analysis software MS-Fit (http://prospector.ucsf.edu/ucsfhtml3.2/msfit.htm) or ProFound (http://canada.proteometrics.com/prowl-cgi/ProFound.exe?FORM =1). Searches were performed in the databases NCBInr and SwissProt. The proteins are referenced by the genbank identifier, accession number and/or version number.

Results. APIT induces either upregulation, downregulation, or modification of the proteins. Modification in the context of this example is a change in the apparent mass and/or the apparent pI value of the protein. By

comparison of 2-DE patterns of APIT-treated whole cell lysates with the corresponding patterns of untreated cells, the proteins as described in Table 3 were identified to be affected by APIT.

Example 10: Transcriptome analysis

The influence of APIT on the gene exression of tumor cells was investigated by Microarray technology.

***In situ* Oligonucleotide Arrays.** A custom oligonucleotide glass array of specific 60mer oligonucleotides representing the mRNA of about 8500 human genes was designed based on human Unigene clusters (Unigene build No. 148) including positive and negative control oligonucleotides (*Homo sapiens* house keeping genes and *Arabadopsis thaliana* genes respectively). The probe design included a base composition filter and a homology search to minimise cross-hybridisation.

RNA isolation, labelling and hybridisation to arrays. Jurkat neo cells (1×10^7 in 20 ml) were cultured for 2 hours in medium (RPMI + 10 % FCS) in the presence or absence of APIT (10 ng/ml) at 37°C, 5% CO_2. Cells were harvested and the pellet was dissolved in 2 ml Trizol (Life Technologies). Total RNA was extracted after addition of chloroform and subsequent centrifugation and precipitated with isopropanol. After washing the pellet with 75% ethanol it was briefly air-dryed. Quality control of the RNA included exclusion of genomic DNA by PCR and "Lab on a chip technology" (Bioanalyser). RNA (5 μg) from each pool was amplified using a reverse transcriptase/T7 polymerase. 1.5 μg of test cRNAs labelled either with Cy3 or Cy5 were hybridised for 16 hours at 65°C to arrays. Each sample was also labelled and hybridised with the reverse fluorophore to obviate possible dye bias. Slides were scanned using a Microarray scanner. Background signal was determined using negative control spots and

subtracted, data were normalised relative to non-regulated genes. Data from duplicate hybridizations were combined.

Results. Table 4 summarizes the genes with increased or decreased transcription rate of treated cells compared with untreated cells, indicating these genes and/or its gene products (proteins) to be targets of APIT and/or H_2O_2.

Claims

1. A polypeptide comprising the amino acid sequence shown in SEQ ID NO:2, 4, or 6.

2. A polypeptide claimed in Claim 1 which is an oxidase which is capable to produce H_2O_2.

3. A polypeptide as claimed in any one of the Claims 1 to 2 which is an alpha amino acid oxidase.

4. A polypeptide as claimed in Claim 3 which is a L-lysine and/or L arginine oxidase.

5. A polypeptide comprising a fragment of the polypeptide as claimed in any one of the Claims 1 to 4.

6. A polypeptide as claimed in Claim 5 which is obtained by protease digestion of the polypeptide as claimed in any of the Claims 1 to 4.

7. A polypeptide as claimed in Claim 6 which is obtained by proteinase K digestion.

8. A polypeptide as claimed in Claim 5 comprising the sequence selected from amino acid residue No. 39 to 77 in SEQ ID NO:2.

9. A polypeptide as claimed in Claim 8 comprising 1 to 20 additional amino acid residues at the N-terminus and/or the C-terminus selected from the sequences of SEQ ID NO:2 or SEQ ID NO:4 adjacent to the sequence selected in claim 8.

10. A polypeptide as claimed in Claim 8 comprising 1 to 10 additional amino acid residues at the N-terminus and/or the C-terminus selected from the sequences of SEQ ID NO:2 adjacent to the sequence selected in claim 8.

11. A polypeptide as claimed in Claim 8 comprising 1 to 5 additional amino acid residues at the N-terminus and/or the C-terminus selected from the sequences of SEQ ID NO:2 adjacent to the sequence selected in claim 8.

12. A polypeptide as claimed in any one of the Claims 2 to 11, wherein the H_2O_2 producing activity can be regulated by the addition or removal of an L-amino acid.

13. A polypeptide as claimed in Claim 12 which is regulated by L-lysine, L-arginine, a derivative or precursor of L-lysine, a derivative or a precursor of L-arginine, or a mixture thereof.

14. A polypeptide which has an identity to the polypeptides of any of the claims 1 to 13 of at least 70%.

15. A polypeptide as claimed in any one of the claims 1 to 14 which is a recombinant polypeptide.

16. The polypeptide as claimed in claim 15, which is a fusion polypeptide.

17. A nucleic acid encoding a polypeptide of any of the Claims 1 to 16.

18. The nucleic acid of Claim 17 comprising
    (a) a nucleotide sequence as shown in SEQ ID NO:1, 3, or 5, or at least the polypeptide coding portion thereof, or the complement thereof, or
    (b) a nucleotide sequence corresponding to the sequence of (a) within the scope of degeneracy of the genetic code, or the complement thereof, or
    (c) a nucleotide sequence hybridizing under stringent condition with the sequence of (a) and/or (b), or
    (d) a nucleotide sequence which has a homology of at least 70% to the sequences of (a) and/or (b).

19. The nucleic acid of claim 17 or 18 operatively linked to an expression control sequence.

20. The nucleic acid of any one of claims 17 to 19 which is a recombinant vector.

21. A recombinant cell comprising the nucleic acid of any one of the Claims 17 to 20.

22. An antibody directed against a polypeptide of any one of the Claims 1 to 16.

23. A pharmaceutical composition or a kit of pharmaceutical compositions comprising the polypeptide as claimed in any of the Claims 1 to 16, in a pharmaceutically effective amount and optionally together with suitable diluents, carriers and/or adjuvants.

24. The pharmaceutical composition or kit of Claim 23 comprising at least one further component which is a substance capable of modulating the cytotoxic activity of the polypeptide.

25. The pharmaceutical composition or kit of Claim 24, wherein the polypeptide and the modulating substances are provided as separate preparations.

26. The pharmaceutical composition or kit of Claim 25, wherein the polypeptide is provided for administration before the modulating substances.

27. The pharmaceutical composition or kit of any one of the Claims 24 to 26, wherein the modulating substance selected from (i) L-lysine, L-arginine, a derivative or precursor of L-lysine, a derivative or precursor of L-arginine, or a mixture thereof, and/or (ii) a flavine nucleoside.

28. The pharmaceutical composition or kit of any one of the Claims 24 to 27, further comprising a nucleic acid, and/or a recombinant cell, and/or an APIT inhibitor.

29. The pharmaceutical composition or kit of Claim 28, wherein the inhibitor is an antibody against the polypeptide.

30. A polypeptide, and/or a nucleic acid, and/or a recombinant cell, and/or an inhibitor as claimed in any one of the Claims 1 to 22, for use in a diagnostic or therapeutic method in humans or animals.

31. A polypeptide, and/or a nucleic acid, and/or a recombinant cell, and/or an inhibitor as claimed in Claim 30 for diagnosis or treatment of cancer.

32. A polypeptide, and/or a nucleic acid, and/or a recombinant cell, and/or an inhibitor as claimed in Claim 30 or 31 for diagnosis or treatment of lung cancer, breast cancer, prostate cancer, colon

cancer, cervix cancer, uterus cancer, larynx cancer, stomach cancer, liver cancer, Ewings sarkoma, acute lymphoid leukemia, chronic myeloid leukemia, apoptosis resistent leukemia, MDR lung cancer, pancreas cancer, gastric cancer, kidney cancer, gliomas, melanomas, chronic lymphoid leukemia, and/or lymphoma.

33. A target substance for a polypeptide of any one of Claim 1-16 as described in Table 3 and/or Table 4.

34. The target substance of Claim 33 which is a protein.

35. The target substance of Claim 33 which is nucleic acid.

36. Use of a target substance of any one of the claims 33 to 35 for the identification of new pharmaceutical agents.

37. Pharmaceutical composition comprising as an active agent at least one of the target substances of any one of claims 33 to 35.

Abstract

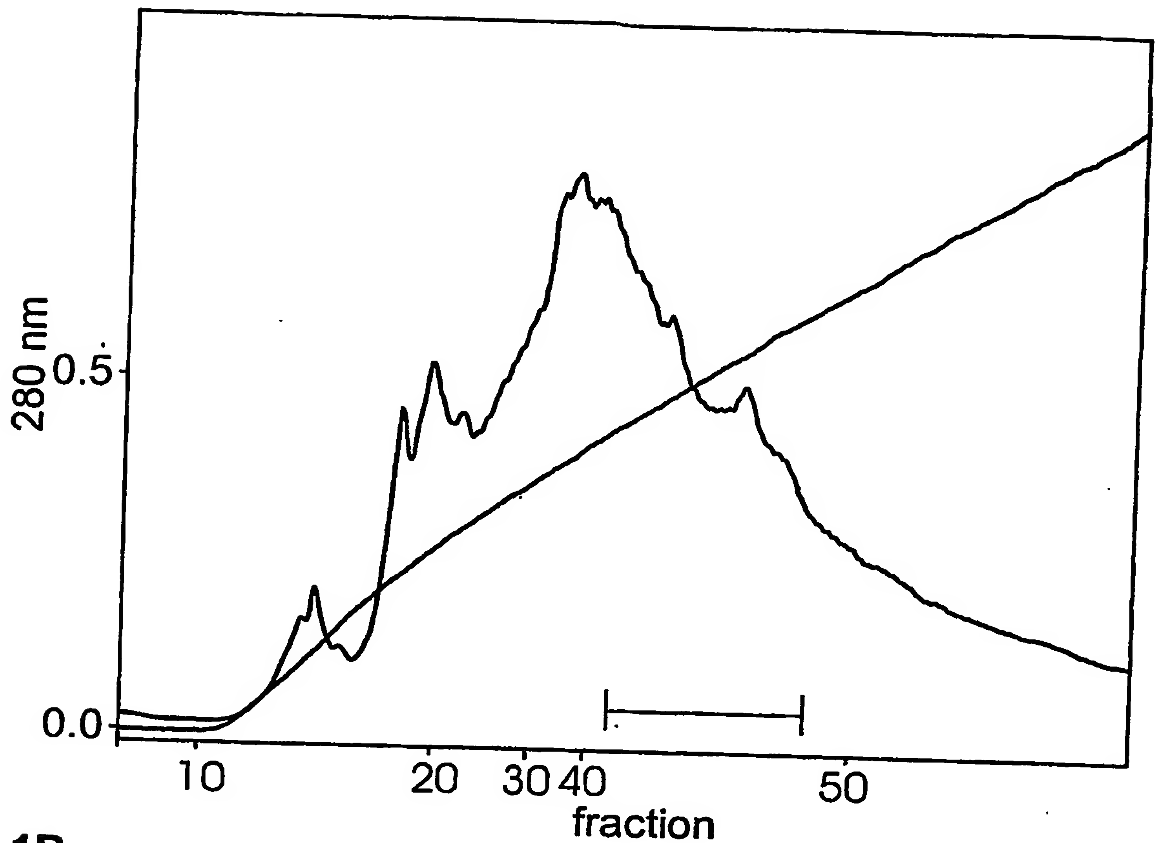
The present invention relates to a cytotoxic polypeptide which is ah L-amino acid oxidase isolated from the ink of the sea hare Aplysia punctata.

ld 20.01.03

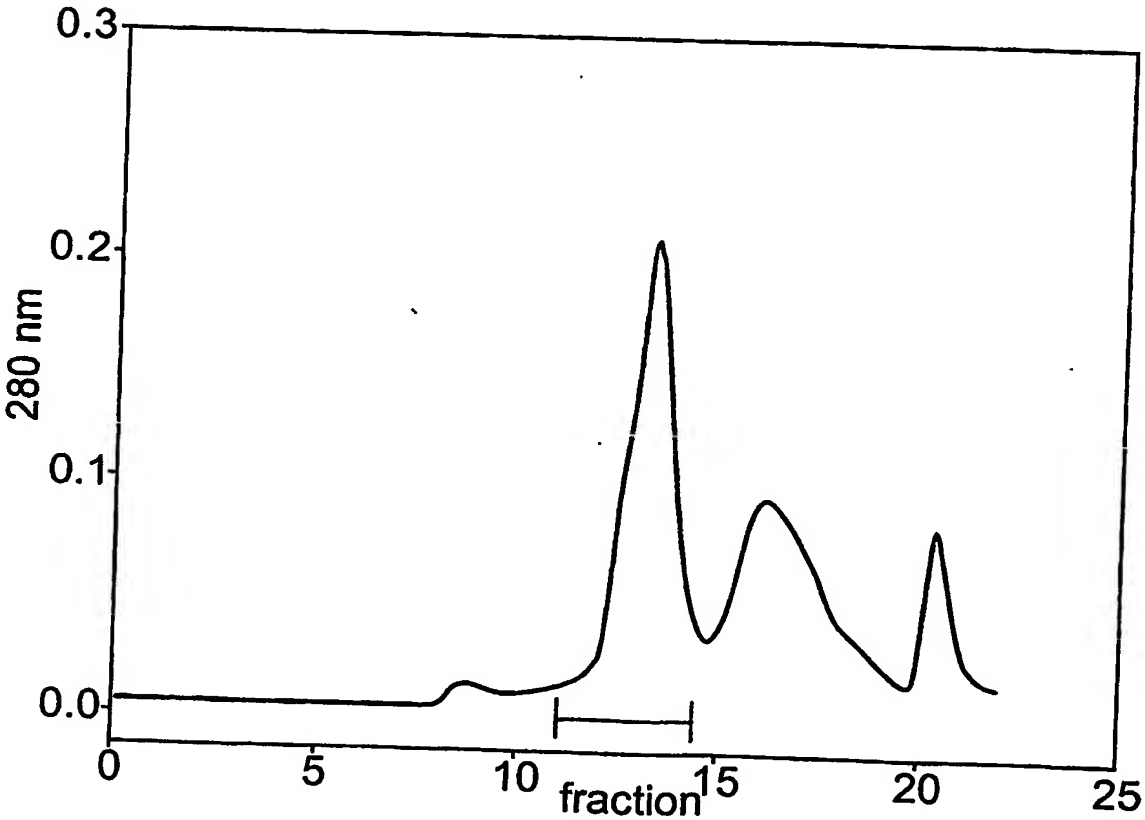
1124

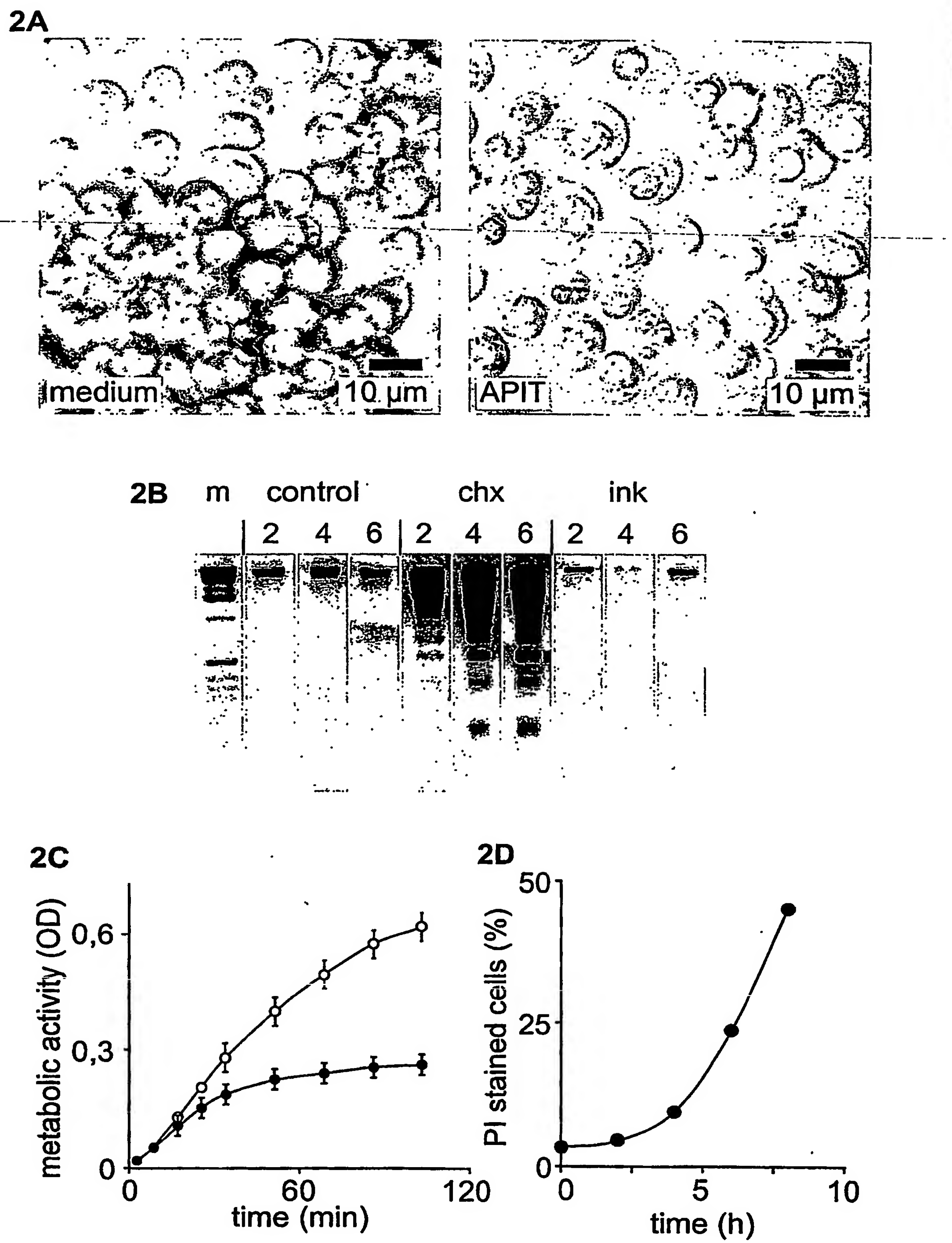
20. Jan. 2003

1A



1B





2A
medium
10 µm
APIT
10 µm
2B
m
control
chx
ink
2
4
6
2
4
6
2
4
6
2C
metabolic activity (OD)
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0
60
120
time (min)
2D
PI stained cells (%)
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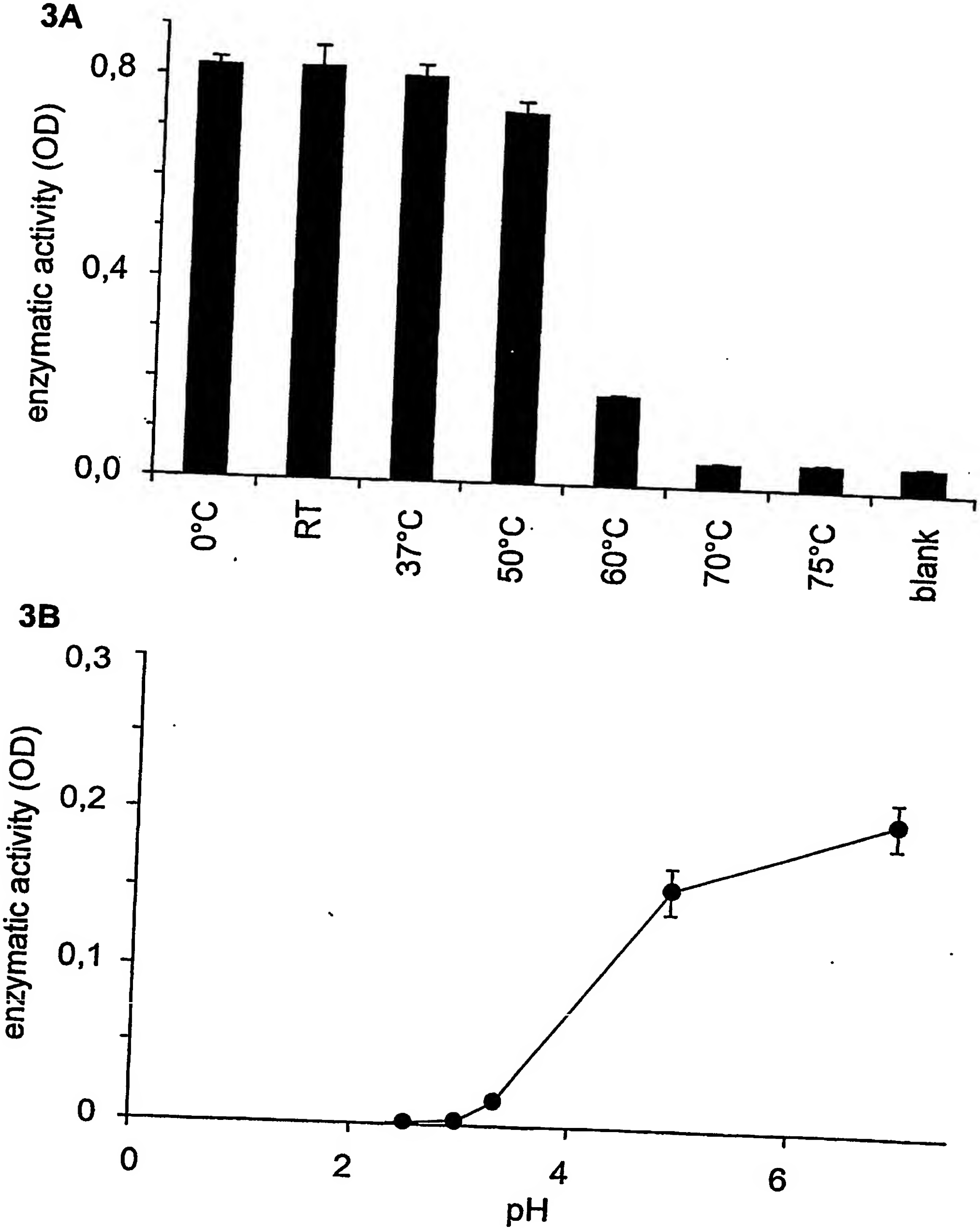
25
0
0
5
10
time (h)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RT
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0,2
0,1
0
0
2
4
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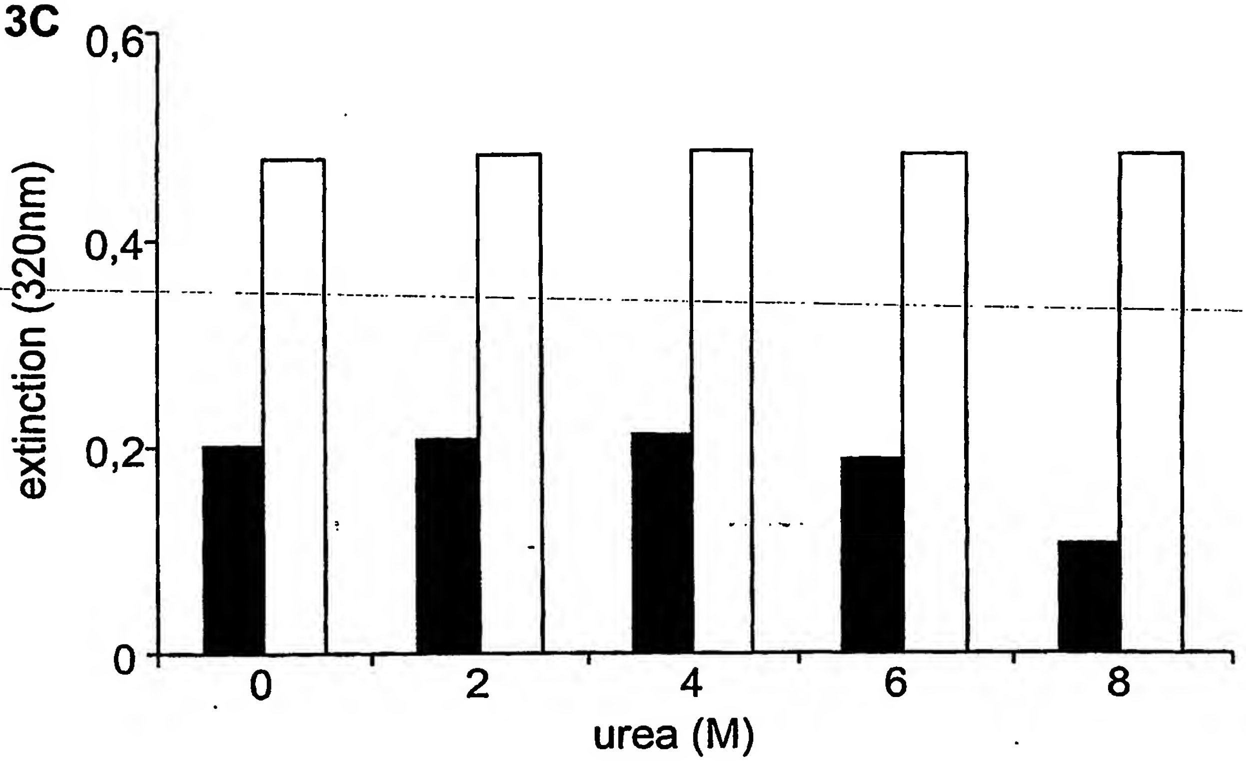
pH

3C



4A

N-terminal sequence: D-G-I-C-R-N-Q-R-Q
Q V R P

Internal peptide sequences

	Sequence
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2	LFXYQLPNTPDVNLEI
3	VISELGLTPK
4	XGDVPYDLSPEEK
5	VILAXPVYALN
6	ATQAYAAVRPIPASK
7	VFMTFDQP
8	SDALFFQMYD
9	SEASGDYILIASYADGLK
10	NQGEDIPGSDPQYNQVTEP(L)(K)

X = not determinable
underlined: primer sequence for RT-PCR

4B

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2	V-Fey 3 DTS 5'	tc gtg ttc gar tac tci gay cg
3	DBuTag1 DTS 3'	ctg tag gtc tag acc tgt tgc a
4	ATF Race 3' 660	ccg tgt aga tct cac tgc cat a
5	Abriged Anchor Primer	ggc cac gcg tcg act agt acg ggi igg gii ggg iig
6	ATF Race 3' 436	ccg ttg agt tgt aga cct
7	AUAP-EcoRI	aatt ggc cac gcg tcg act agt ac
8	ATF 5' Sign Eco RI GEX/ET	aa ttc tcg tct gct gtg ctt ctc ct
9	ATF 3' XhoI	gac tta gag gaa gta gtc gtt ga

4C

```
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...TCGTCTGCTGTGCTTCTCCTGGCTTGTGCGTTGGTCATCTCTGTCCACGCCGACGGTGTCTGC
.....................................................GACGGTATCTGC

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4C (continued)

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D Y Y G S E I Y T L K E G L S S V P Q G L L
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GACTACTACGGCAGTGAGATCTACACCCTCAAGGAAGGACTGTCTTCCGTCCCTCAAGGGCTCCTA
GACTACTATGGCAGTGAGATCTACACCCTAAAGGAAGGACTGTCTTCCGTCCCACAAGGGCTCCTA

Q AT F L D A A D S N E F Y P N S H L K A L R
CAGGCTTTTCTGGACGCCGCAGACTCCAACGAGTTCTATCCCAACAGCCACCTGAAGGCCCTGAGA
CAGGCTTTTCTGGACGCCGCAGACTCCAACGAGTTCTATCCCAACAGCCACCTGAAGGCCCTGAGA
CAGACTTTTCTGGACGCCGCAGACTCGAACGAGTTCTATCCCAACAGCCACCTGAAGGCCCTGAGA

R K T N G Q Y V L Y F E P T T S K D G Q T T
CGTAAGACCAACGGTCAGTATGTTCTTTACTTTGAGCCCACCACCTCCAAGGATGGACAAACCACA
CGTAAGACCAACGGTCAGTATGTTCTTTACTTTGAGCCCACCACCTCCAAGGATGGACAAACCACA
CGTAAGACCAACGGTCAGTATGTTCTTTACTTTGAGCCCACCACCTCCAAGGATGGACAAACCACA

I N Y L E P L Q V V C A Q R V I L A M P V Y
ATCAACTATCTGGAACCCCTGCAGGTTGTGTGTGCACAAAGAGTCATCCTGGCCATGCCGGTATAC
ATCAACTATCTGGAACCCCTGCAGGTTGTGTGTGCACAGAGAGTCATTCTGGCCATGCCGGTCTAC
ATCAACTATCTGGAACCCCTGCAGGTTGTGTGTGCACAGAGAGTCATCCTGGCCATGCCGGTCTAC

A L N Q L D W N Q L R N D R A T Q A Y A A V
GCTCTGAACCAACTAGACTGGAATCAGCTCAGAAATGACCGAGCCACCCAAGCGTACGCTGCCGTT
GCTCTCAACCAGTTGGATTGGAATCAGCTCAGAAATGACCGAGCCACCCAAGCGTACGCTGCCGTG
GCTCTCAACCAACTGGACTGGAATCAGCTCAGAAATGACCGAGCCACCCAAGCGTACGCTGCCGTG

R P I P A S K V F M TS F D Q P W W L E N E R
CGCCCGATTCCTGCAAGTAAGGTGTTCATGTCCTTTGATCAGCCCTGGTGGTTGGAGAACGAGAGG
CGCCCGATTCCTGCAAGTAAGGTGTTCATGACCTTTGATCAGCCCTGGTGGTTGGAGAACGAGAGG
CGCCCGATTCCTGCAAGTAAAGTGTTCATGACCTTTGATCAGCCCTGGTGGTTGGAGAACGAGAGG

K S W V T K S D A L F S Q M Y D W Q K S E A
AAATCCTGGGTCACCAAGTCGGACGCGCTTTTCAGCCAAATGTACGACTGGCAGAAGTCTGAGGCG
AAATCCTGGGTCACCAAGTCGGACGCGCTTTTCAGTCAAATGTACGACTGGCAGAAGTCTGAGGCG
AAATCCTGGGTCACCAAGTCGGACGCGCTTTTCAGCCAAATGTACGACTGGCAGAAGTCTGAGGCG

S G D Y I L I A S Y A D G L K A Q Y L R E L
TCCGGAGACTACATCCTGATCGCCAGCTACGCCGACGGCCTCAAAGCCCAGTACCTGCGGGAGCTG
TCCGGAGACTACATCCTGATCGCCAGCTACGCCGACGGCCTCAAAGCCCAGTACCTGCGGGAGCTG
TCCGGAGACTACATCCTGATCGCCAGCTACGCCGACGGCCTCAAAGCCCAGTACCTGCGGGAGCTG

K N Q G E D I P G S D P G Y N Q V T E P L K
AAGAATCAGGGAGAGGACATCCCAGGCTCTGACCCAGGCTACAACCAGGTTACCGAACCCCTCAAG
AAGAATCAGGGAGAGGACATCCCAGGCTCTGACCCAGGCTACAACCAGGTCACCGAACCCCTCAAG
AAGAATCAGGGAGAGGACATCCCAGGCTCTGACCCAGGCTACAACCAGGTCACCGAACCCCTCAAG
```

4C (continued)

```
 D  T  I  L  D  H  L  T  E  A  Y  G  V  E  R  D  S  I  PR  E  P  V
GACACCATTCTTGACCACCTCACTGAGGCTTATGGCGTGGAGCGAGACTCGATCCCGGAACCCGTG
GACACCATTCTTGACCACCTCACTGAGGCCTATGGCGTGGAGCGAGACTCGATCCCGGAACCCGTG
GACACCATTCTTGACCACCTCACTGAGGCTTATGGCGTGGAACGAGACTCGATCCGGGAACCCGTG

 T  A  A  S  Q  F  W  T  D  Y  P  F  G  C  G  W  I  T  W  R  A  G
ACCGCCGCTTCCCAGTTCTGGACAGACTACCCGTTTGGCTGTGGATGGATCACCTGGAGGGCCGGC
ACCGCCGCTTCCCAGTTCTGGACAGACTACCCGTTTGGCTGTGGATGGATCACCTGGAGGGCCGGC
ACCGCCGCTTCCCAGTTCTGGACCGACTACCCGTTCGGCTGTGGATGGATCACCTGGAGGGCAGGC

 F  H  F  D  D  V  I  S  T  M  R  R  P  S  L  K  D  E  V  Y  V  V
TTCCATTTCGATGACGTCATCAGCACCATGCGTCGCCCGTCACTGAAAGATGAGGTCTACGTGGTG
TTCCATTTCGATGACGTCATCAGCACCATGCGTCGCCCGTCACTGAAAGATGAGGTATACGTGGTG
TTCCATTTTGATGACGTCATCAGCACCATGCGTCGCCCGTCACTGAAAGATGAGGTCTACGTGGTG

 G  A  D  Y  S  W  G  L  I  S  S  W  I  E  G  A  L  E  T  S  E  N
GGAGCCGACTACTCCTGGGGACTTATCTCCTCCTGGATAGAGGGCGCTCTGGAGACCTCGGAAAAC
GGAGCCGATTACTCCTGGGGACTTATCTCCTCCTGGATAGAGGGCGCTCTGGAGACCTCGGAAAAC
GGAGCCGATTACTCCTGGGGACTTATCTCCTCCTGGATAGAGGGCGCTCTGGAGACCTCAGAAAAC

 V  I  N  D  Y  F  L  -
GTCATCAACGACTACTTCCTCTAA
GTCATCAACGACTACTTCCTCTAA
GTCATCAACGACTACTTCCTCTAA
```

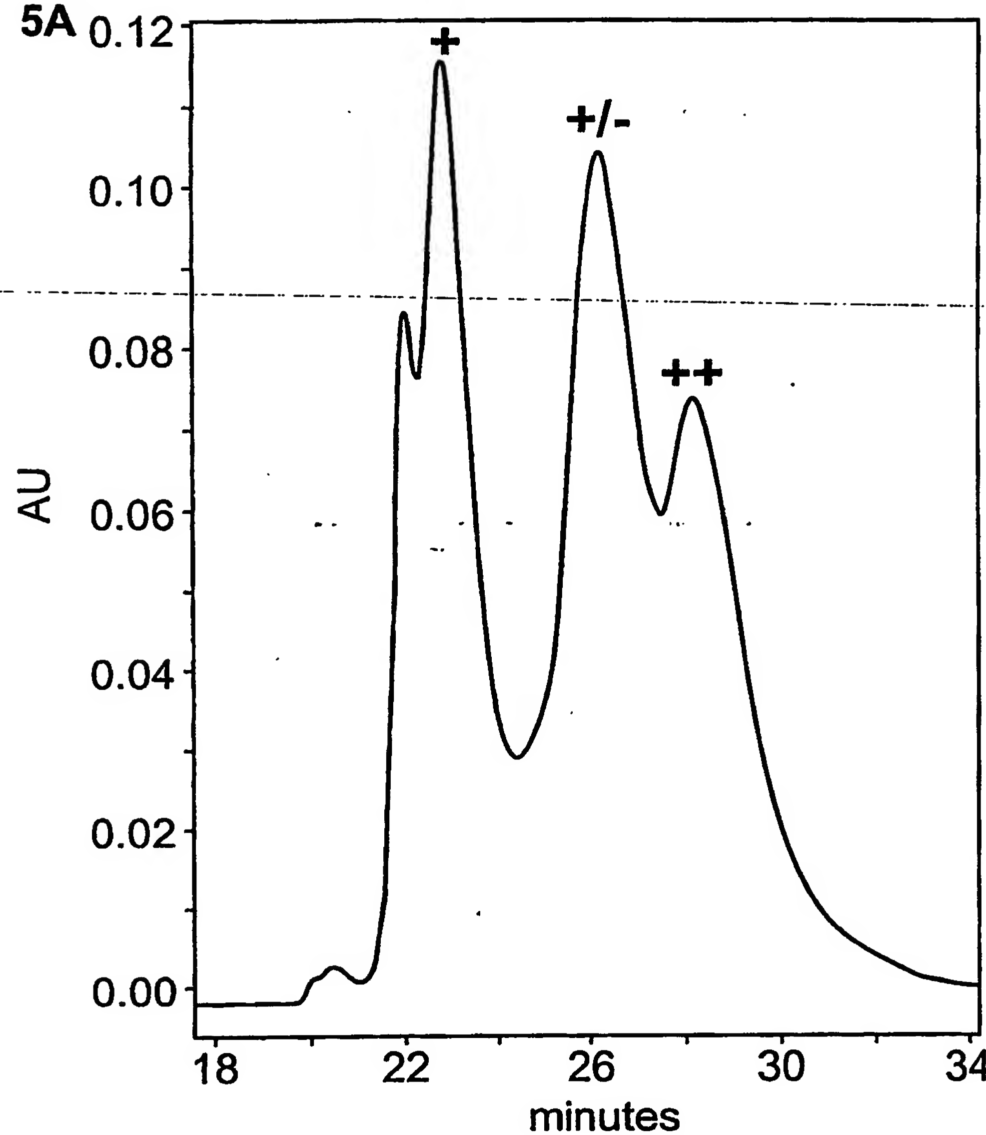
4D

```
                          VS
MSSAVLLLACALVISVHADGICRNRRQCNREVCGSTYD[VAVVGA
        ^         ^         ^         ^
       10        20        30        40

     T         Q         H         S
GPGGANSAYMLRDSGLDIAVFE]YSD[RVGGRLFT]YQLPNTPDVNL
     ^         ^         ^         ^
    50        60        70        80
```

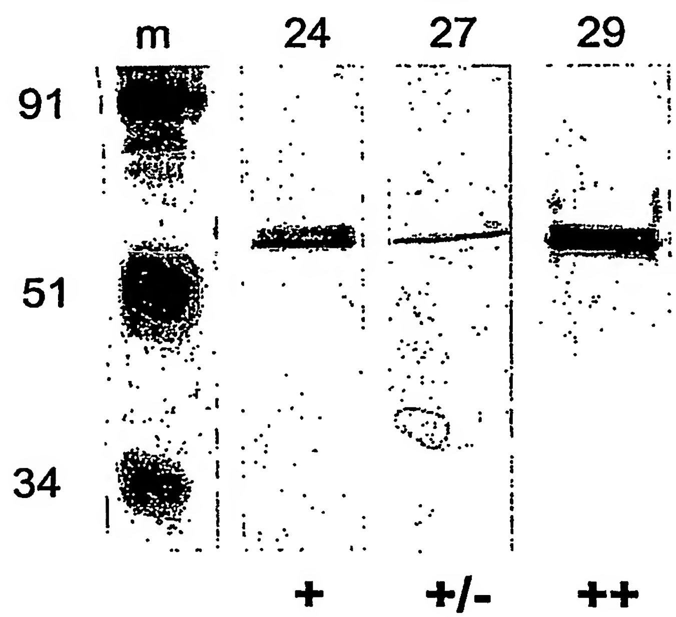
10124

5A



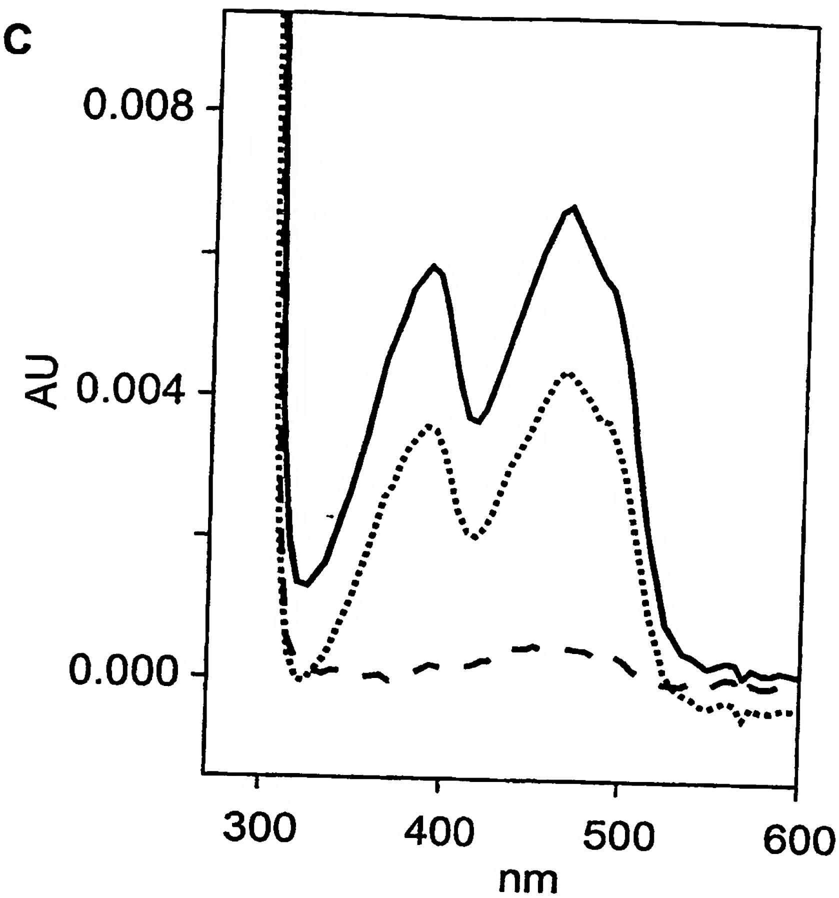
+
+/-
++
0.12
0.10
0.08
0.06
0.04
0.02
0.00
AU
18
22
26
30
34
minutes

5B

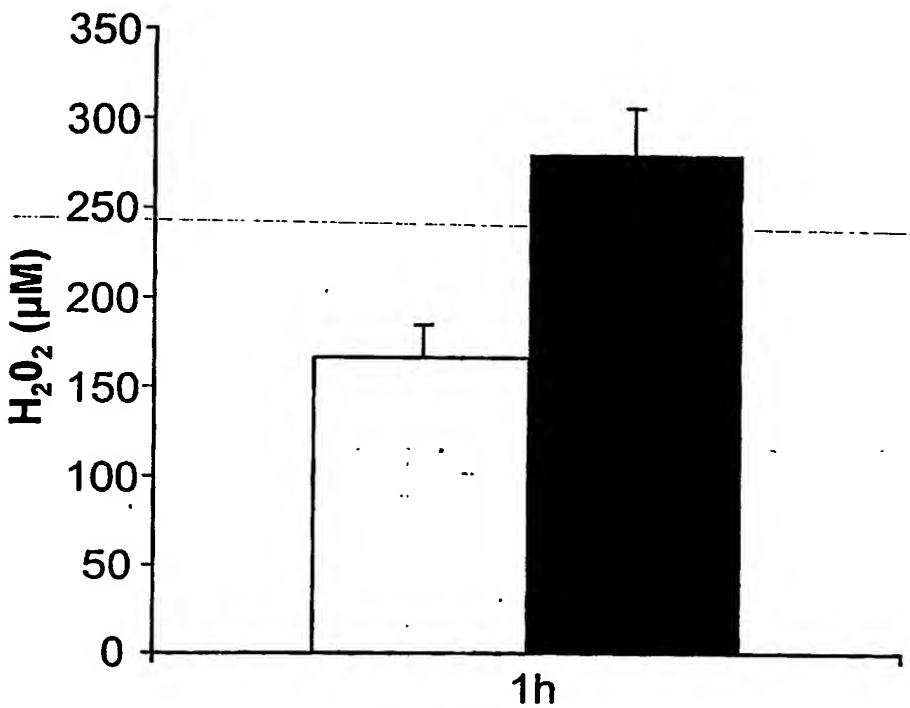


m
24
27
29
91
51
34
+
+/-
++

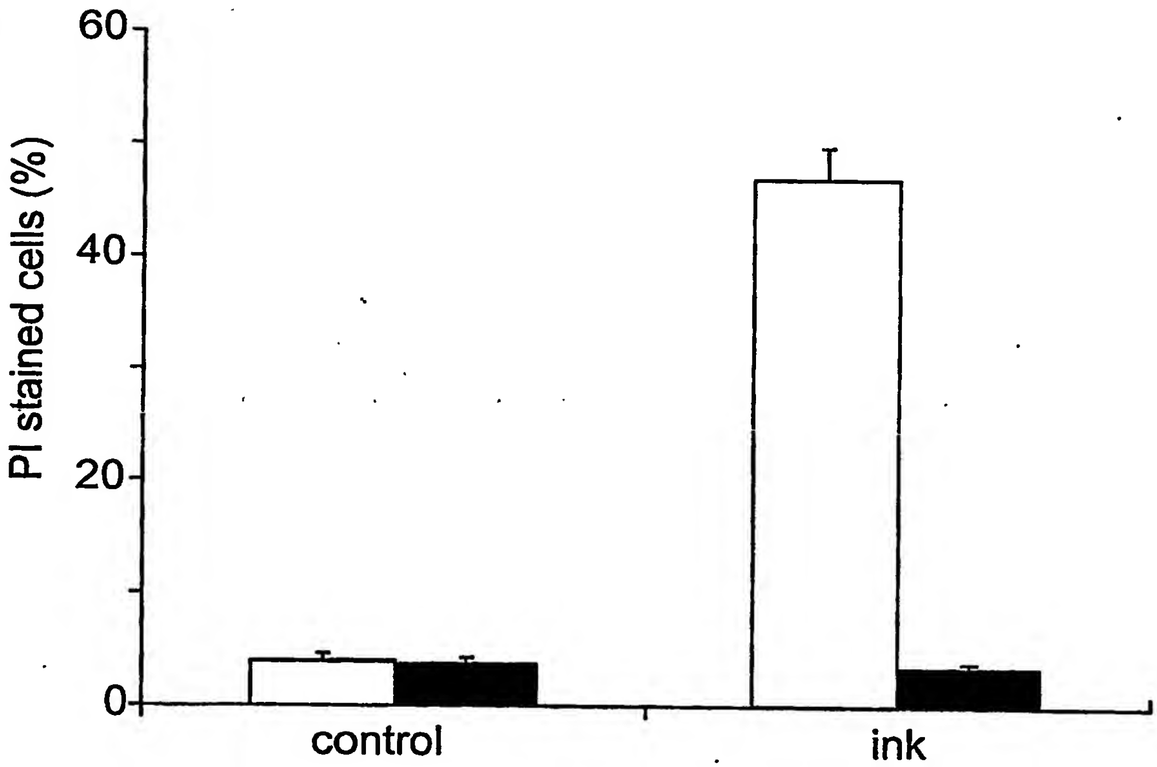
5 C



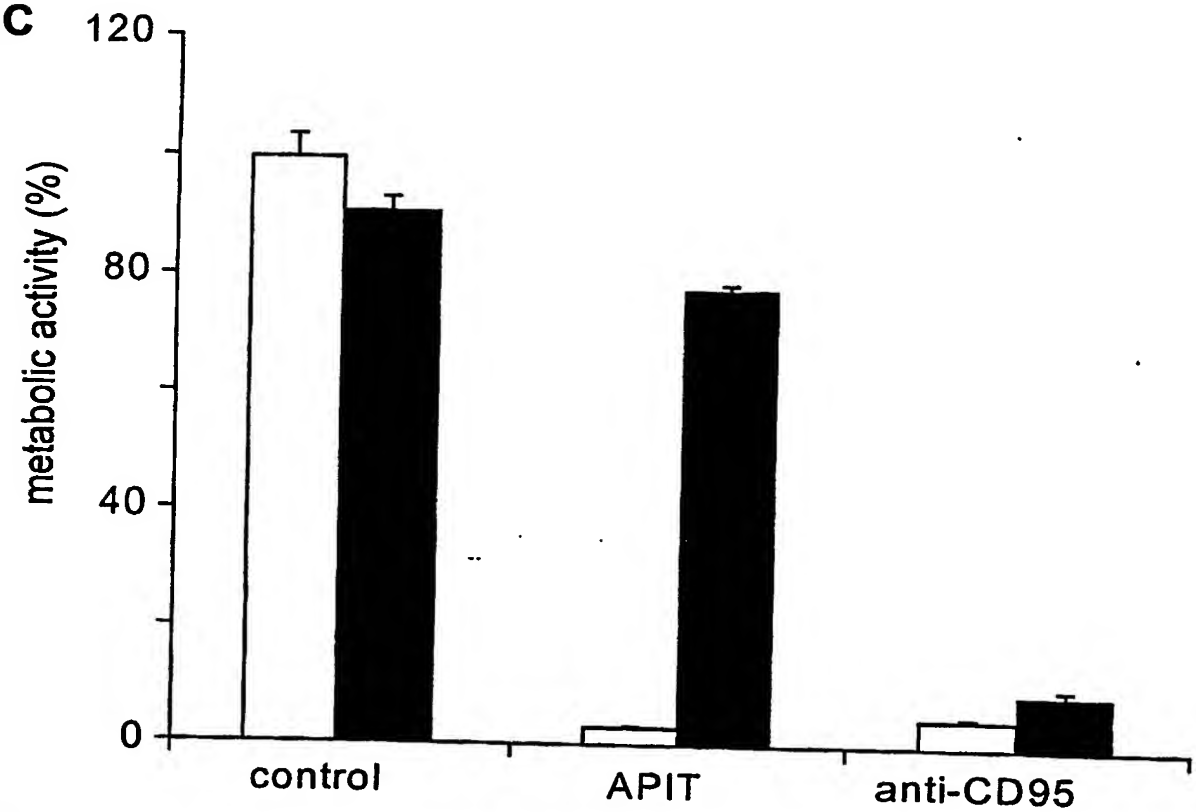
6A


350
300
250
200
150
100
50
0
H_2O_2 (µM)
1h

6B


60
40
20
0
PI stained cells (%)
control
ink

6C


120
80
40
0
metabolic activity (%)
control
APIT
anti-CD95

6D

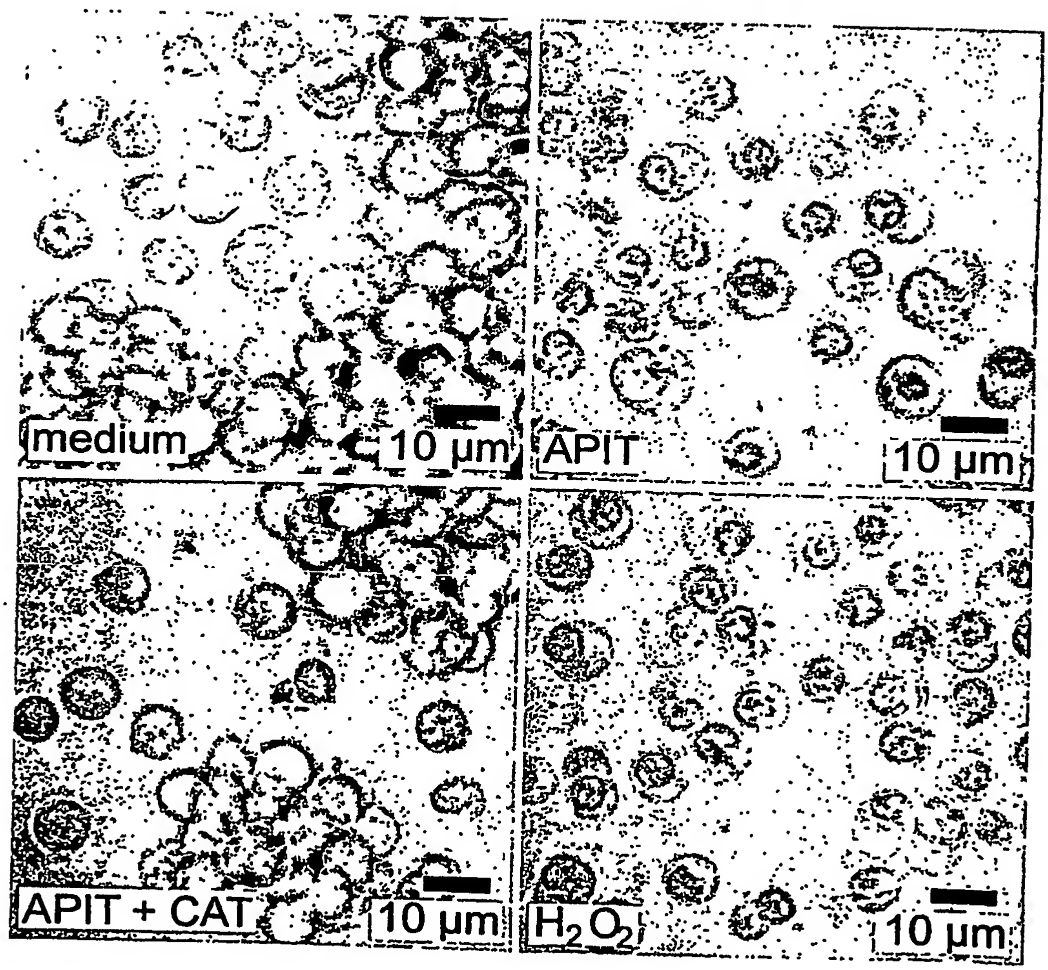
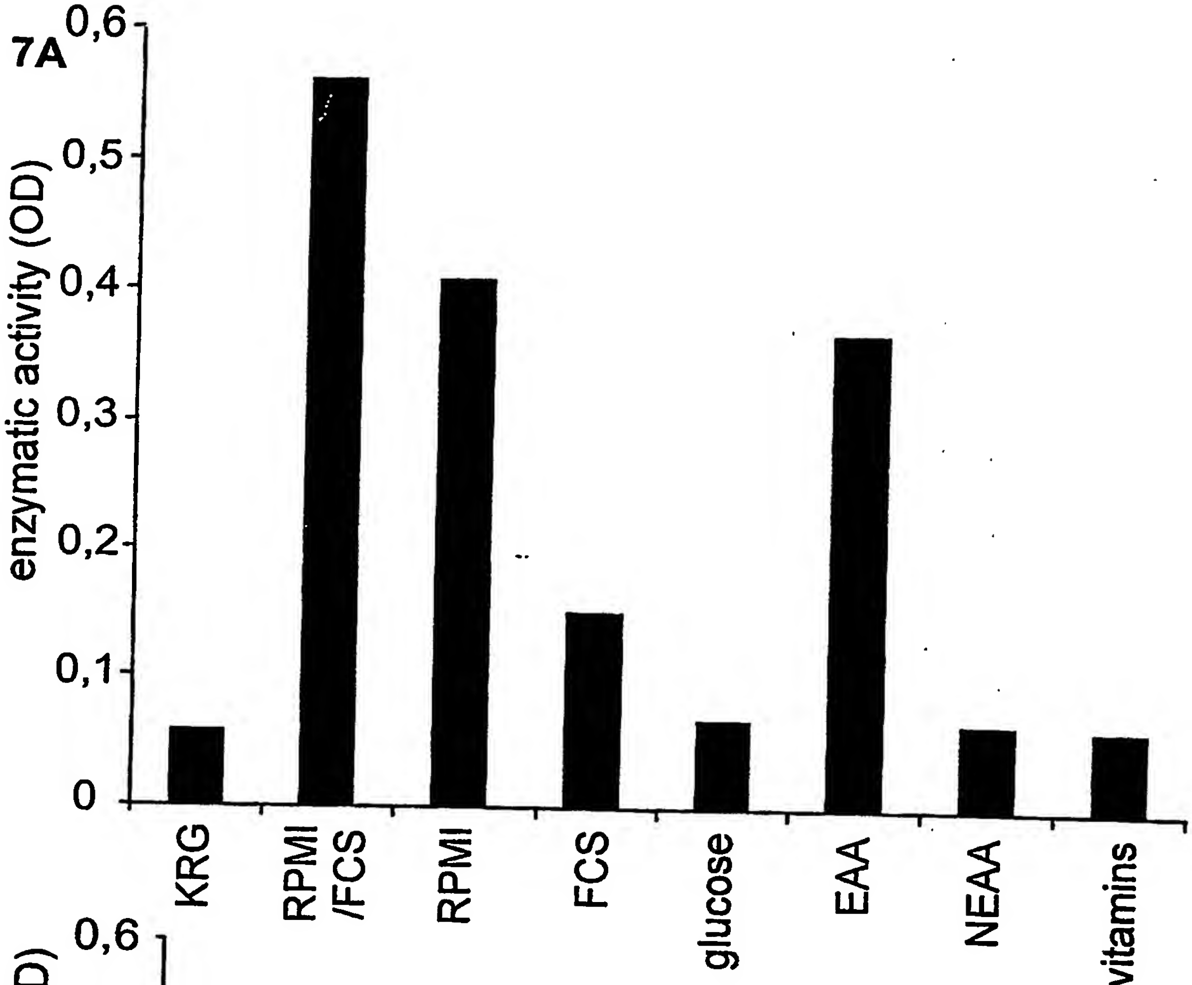

medium
10 µm
APIT
10 µm
APIT + CAT
10 µm
H_2O_2
10 µm

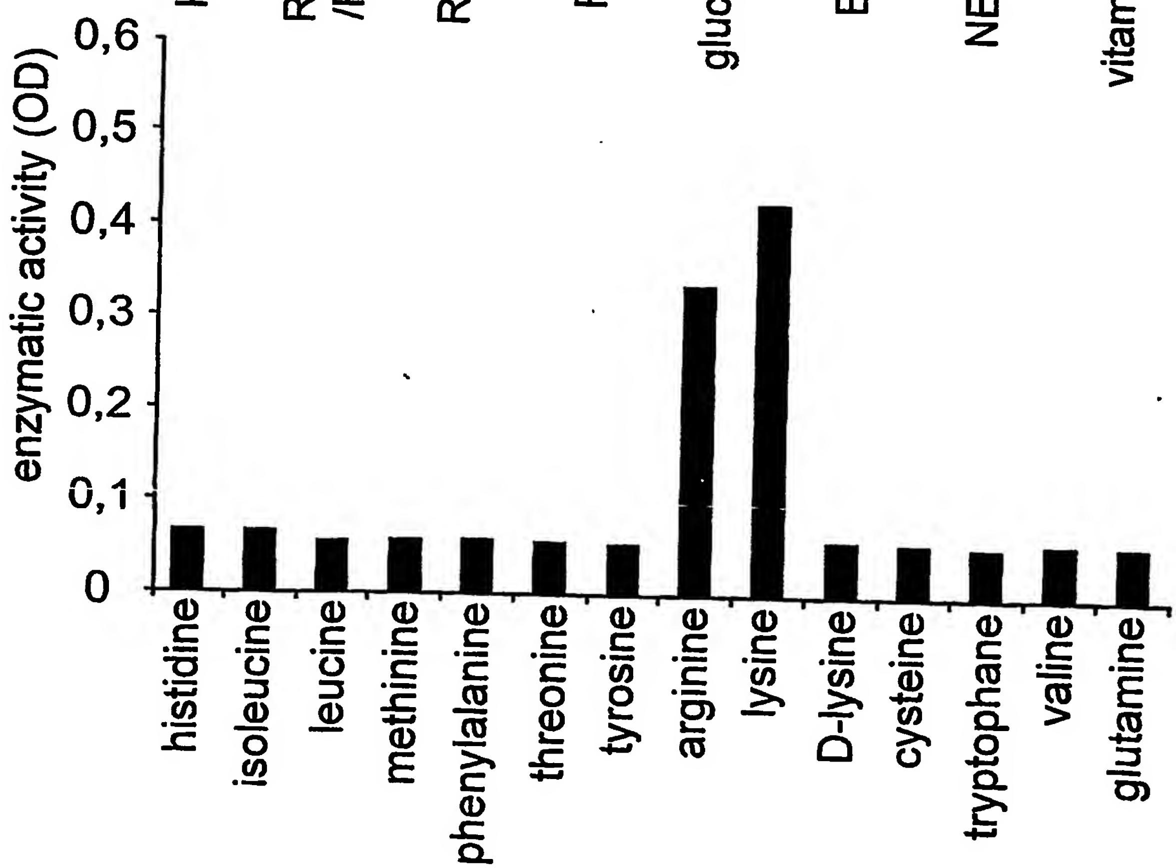
Table 1

EAA (essential amino acids)		**NEAA** (non-essential aino acids)	
L-arginine·HCl	126.4 mg/l	L-alanine	8.9 mg/l
L-cystine	24.02 mg/l	L-asparagine	13.2 mg/l
L-histidine·HCl·H2O	41.92 mg/l	L-aspartic-acid	13.3 mg/l
L-isoleucine	52.46 mg/l	L-glutamic acid	14.7 mg/l
L-leucine	52.46 mg/l	glycine	7.5 mg/l
L-lysine·HCl	73.06 mg/l	L-prolin	11.5 mg/l
L-methionine	14.92 mg/l	L-serine	10.5 mg/l
L-phenylalanine	33.02 mg/l		
L-threonine	47.64 mg/l		
L-tryptophane	10.2 mg/l		
L-tyrosine	36.22 mg/l		
L-valine	46.86 mg/l		

Single amino acids	
histidine·HCl·H2O	20 mg/l
isoleucine	50 mg/l
leucine	50 mg/l
methionine	15 mg/l
phenylalanine	15 mg/l
threonine	20 mg/l
tyrosine	20 mg/l
arginine·HCl	240 mg/l
lysine	40 mg/l
D-lysine	40 mg/l
cystine	50 mg/l
tryptophane	5 mg/l
valine	20 mg/l
glutamine	300 mg/l

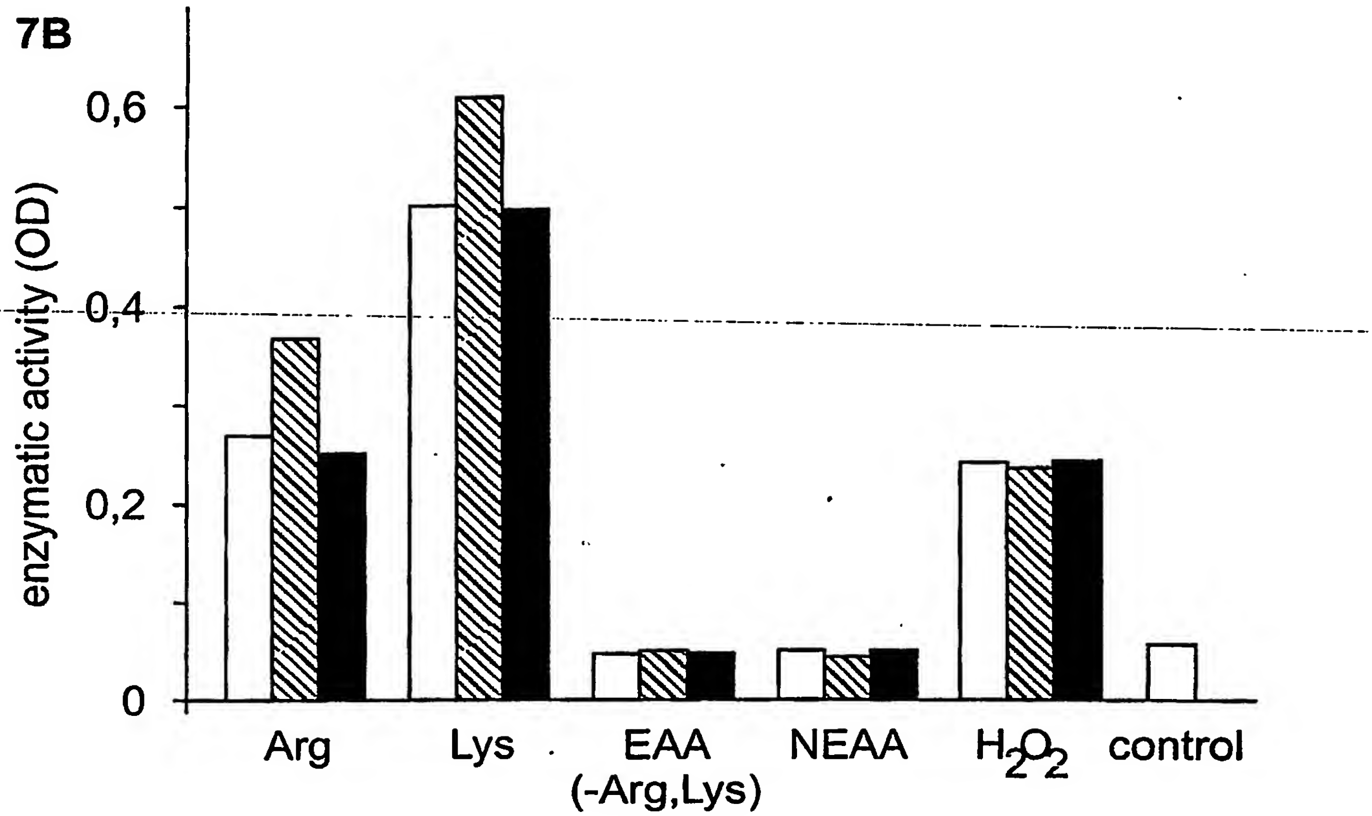
7A


enzymatic activity (OD)
0,6
0,5
0,4
0,3
0,2
0,1
0
KRG
RPMI /FCS
RPMI
FCS
glucose
EAA
NEAA
vitamins


enzymatic activity (OD)
0,6
0,5
0,4
0,3
0,2
0,1
0
histidine
isoleucine
leucine
methinine
phenylalanine
threonine
tyrosine
arginine
lysine
D-lysine
cysteine
tryptophane
valine
glutamine

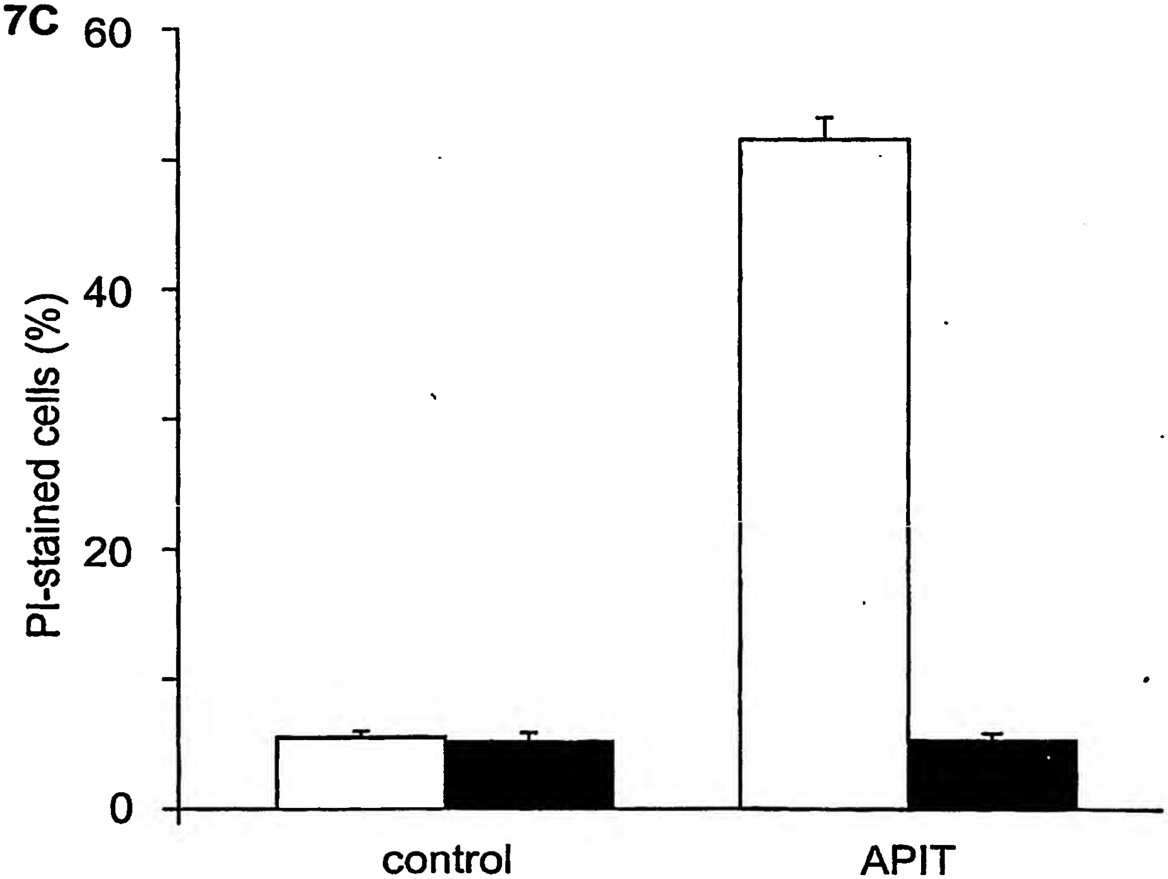
16124

7B



0,6
0,4
0,2
0
enzymatic activity (OD)
Arg
Lys
EAA
(-Arg,Lys)
NEAA
H2O2
control

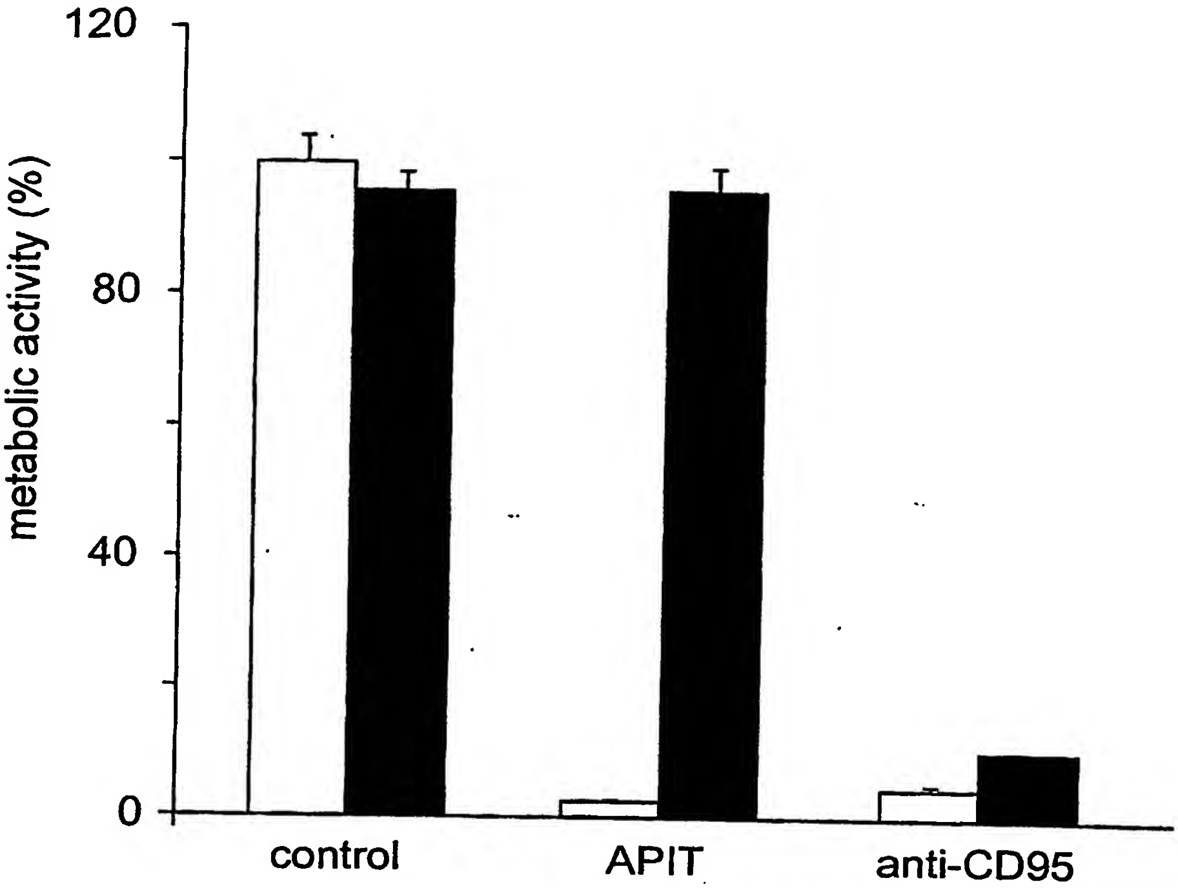
7C



60
40
20
0
PI-stained cells (%)
control
APIT

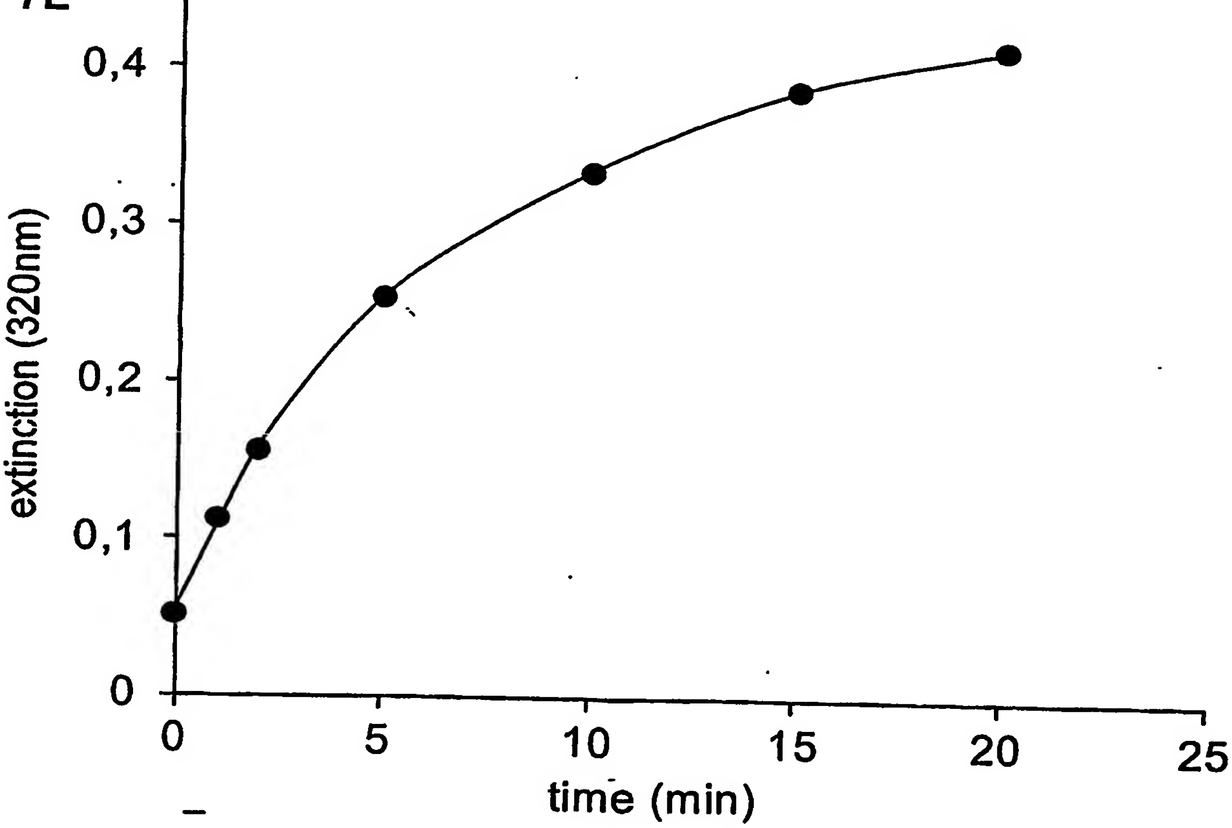
17124

7D



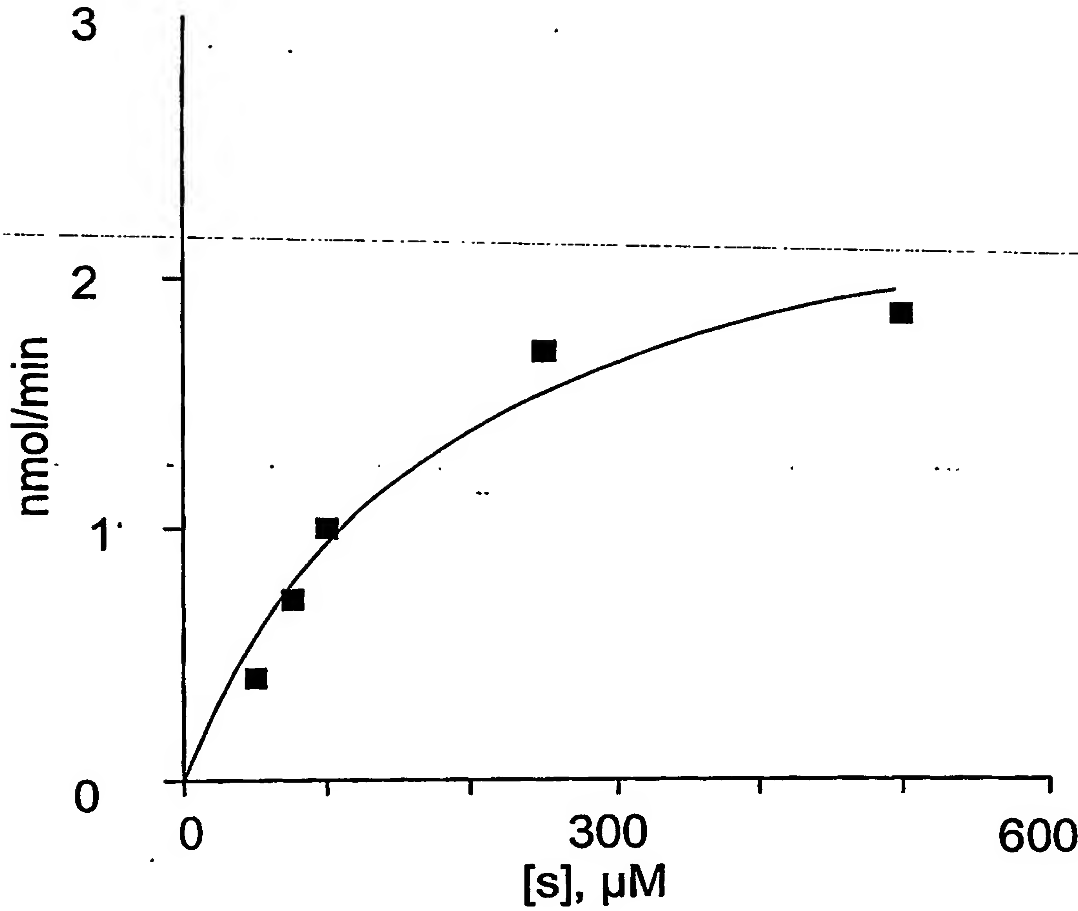
120
80
40
0
metabolic activity (%)
control
APIT
anti-CD95

7E

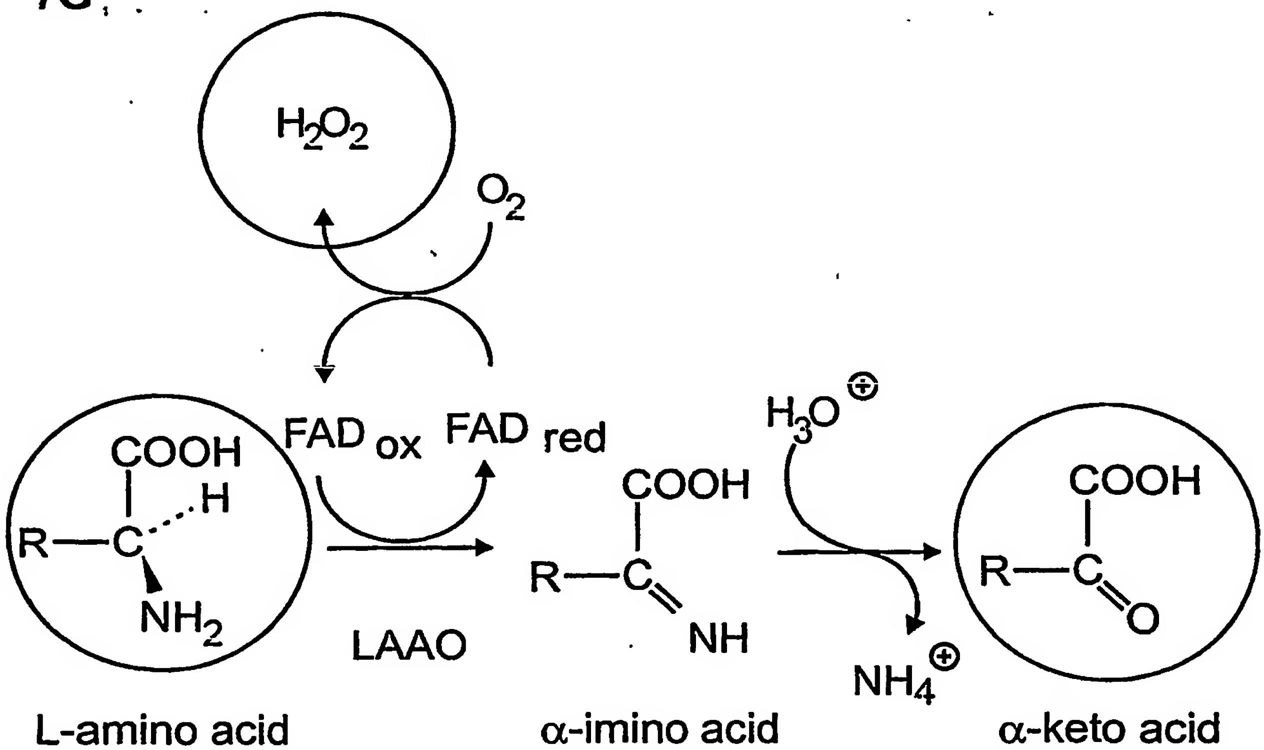


0,4
0,3
0,2
0,1
0
extinction (320nm)
0
5
10
15
20
25
time (min)

7F


3
2
1
0
nmol/min
0
300
600
[s], μM

7G


H2O2
O2
FAD ox
FAD red
H3O⊕
COOH
H
R—C
NH2
LAAO
COOH
R—C=NH
NH4⊕
COOH
R—C=O
L-amino acid
α-imino acid
α-keto acid

19124

Table 2. APIT kills different tumor cell lines

models for	kind of tumor	tumor cell line	IC50 (ng/ml)
1. solid tumors	lung cancer	GLC4	9
	breast cancer	MCF-7, SK-BR-3	*
	prostate cancer	PC3, DU145	*
	colon cancer	HT-29	20
	cervix cancer	HeLa, Chang	*, 10
	uterus carcinoma	Hec-1-B	*
	larynx cancer	HEp-2	*
	stomach cancer	AGS	*
	liver cancer	Hep G2	*
2. leukemia	T cell leukemia (ALL)	Jurkat neo	3.2
	T cell leukemia (ALL)	CEM neo	5.6
	B cell leukemia	SKW neo	3
	Monocyte leukemia (AML)	Mono Mac 6	*
	Monocyte leukemia (AML)	THP-1	10
3. "orphan" tumors	Ewings sarcoma	RDES	4.5
		A673	5
4. apoptosis resistant tumors	(CML)	K562	4.25
	T cell leukemia (ALL)	Jurkat Bcl-2	2.7
	T cell leukemia (ALL)	CEM Bcl-X_L	4.0
	B cell leukemia	SKW Bcl-2	5.5
5. MDR tumors	Lung cancer	GLC4-ADR	10

20124

Table 3. Proteome analysis

Description	gi	NCBI	NCBI version	swissprot	effect
Aldolase A (E.C.4.1.2.13)	229674	1ALD	1ALD	P04075	-
26S proteasome regulatory chain 12	2134660	S65491	S65491	-	-
3-Hydroxyacyl-CoA dehydrogenase	2078327	AAB54008	AAB54008.1	Q16836	-
C-1-tetrahydrofolate synthase, cytoplasmic (C1-THF synthase)	115206	P11586	P11586	P11586	-
Chain A, Structure Of Human Glutamate Dehydrogenase-Apo Form *or* Glutamate dehydrogenase 1	20151189 4885281	1L1F_A NP_005262	1L1F_A NP_005262.1	- P00367	m
Cleavage and polyadenylation specific factor 5, 25 kD subunit	5901926	NP_008937	NP_008937.1		+
Cofilin 1	5031635	NP_005498	NP_005498.1	P23528	-
Coronin, actin binding protein, 1A	5902134	NP_009005	NP_009005.1	P31146	+
Dihydrolipoamide dehydrogenase precursor; E3 component of pyruvate dehydrogenase	4557525	NP_000099	NP_000099.1	P09622	-
dJ553F4.4 (Novel protein similar to Drosophila CG8055 protein)	12314022	CAC14088	CAC14088.1		+
DNA replication licensing factor MCM4	1705520			P33991	+
Elongation factor1-delta (EF-1-delta)	20141357	P29692	P29692	P29692	-
Enolase 1, alpha; phosphopyruvate hydratase	4503571	NP_001419	NP_001419.1	Q05524	+
Glyceraldehyde-3-phosphate dehydrogenase *or* uracil DNAglycosylase	31645 35053	CAA25833 CAA37794	CAA25833.1 CAA37794.1	P04406* P04406*	+
Heat shock 60kD protein 1 (chaperonin)	14603309	AAH10112	AAH10112.1	Q96FZ6	-
Heat shock 60kDa protein 1 (chaperonin)	4504521	NP_002147	NP_002147.1	P10809	-
Heat shock 70kD protein 9B (mortalin-2)	4758570	NP_004125	NP_004125.1	Q8N1C8	-
Heterogeneous nuclear ribonucleoprotein C, isoform b	4758544	NP_004491	NP_004491.1	P07910	m
Hspc117	6841456	AAF29081	AAF29081.1	Q9P037	m
Inosine-5'-monophosphate dehydrogenase 2 (IMP dehydrogenase 2)	124419	P12268	P12268	P12268	+
Isocitrate dehydrogenase 3 (NAD+) alpha	5031777	NP_005521	NP_005521.1	P50213	-
KH-type splicing regulatory protein (FUSE binding protein 2)	4504865	NP_003676	NP_003676.1	-	-
Nuclear matrix protein NMP200 related to splicing factor PRP19	7657381	NP_055317	NP_055317.1	Q9UMS4	-
Nucleobindin 2	4826870	NP_005004	NP_005004.1	P80303	-
54 kDa nuclear RNA- and DNA-binding protein (p54(nrb)) (p54nrb)	13124797	Q15233	Q15233	Q15233	+
Peroxiredoxin 1 (Thioredoxin peroxidase 2)	548453	Q06830	Q06830	Q06830	m
Peroxiredoxin 1; Proliferation-associated gene A; proliferation-associated gene A	4505591	NP_002565.1	NP_002565.1	Q06830	m

21124

Table 3. Continuation I

Description	gi	NCBI	NCBI version	swissprot	effect
Peroxiredoxin 2 (Thioredoxin peroxidase 1)	2507169	P32119	P32119	P32119	+
Peroxiredoxin 3; antioxidant protein 1; thioredoxin-dependent peroxide reductase precursor	5802974	NP_006784	NP_006784.1	P30048	-
2-phosphopyruvate-hydratase alpha-enolase; carbonate dehydratase	693933	CAA59331	CAA59331.1	P06733	+
Proteasome subunit alpha type 7	12643540	O14818	O14818	O14818	+
Proteasome subunit beta type 1 (Proteasome component C5) (Macropain subunit C5)	130853	P20618	P20618	P20618	+
Ras-GTPase-activating protein SH3-domain-binding protein; GAP binding protein	5031703	NP_005745.1	NP_005745.1	Q13283	m
Replication protein A2, 32kDa	4506585	NP_002937	NP_002937.1	P15927	-
Rho GDP-dissociation inhibitor 2 (Rho GDI 2) (Rho-GDI beta) (Ly-GDI)	1707893	P52566	P52566	P52566	-
Ribosomal protein P0; 60S acidic ribosomal protein P0	4506667	NP_000993	NP_000993.1	P05388	-
or similar BLOCK 23	20536934	XP_165448	XP_165448.1	Q8NHW5	
Ribosomal protein, large, P0	12654583	AAH01127	AAH01127.1	P05388	-
RNA-binding protein regulatory subunit	6005749	NP_009193	NP_009193.1	O14805	+
RNA-binding protein regulatory subunit	12720028	XP_001707	XP_001707.2	O14805	+
Semenogelin I; Semenogelin	4506883	NP_002998	NP_002998.1	P04279	-
Similar to villin 2 (ezrin)	15530243	AAH13903	AAH13903.1	P15311	-
Splicing factor proline/glutamine rich (polypyrimidine tract binding protein associated)	4826998	NP_005057	NP_005057.1	P23246	-
Stathmin 1; metablastin; prosolin; oncoprotein 18; phosphoprotein 19; leukemia-associated phosphoprotein p18	5031851	NP_005554	NP_005554.1		-
U2 small nuclear ribonucleoprotein A' (U2 snRNP-A')	134094	P09661	P09661	P09661	+
Vimentin	4507895	NP_003371	NP_003371.1	P08670	-
Voltage-dependent anion-selective channel protein 2 (VDAC-2) (hVDAC2)	1172554	P45880	P45880	P45880	-

Table 4. Transcriptome analysis

Unigene cluster	Description	GENE	gi	pir/NCBI/swissp	effect
Hs.3833	3'-phosphoadenosine 5'-phosphosulfate synthase 1	PAPSS1	4885537	NP_005434.1	-
Hs.166563	replication factor C (activator 1) 1, 145kDa	RFC1	15011931	ref:NP_002904.2	-
Hs.78991	DNA segment, numerous copies, expressed probes (GS1 gene)	DXF68S1E	6912346	ref:NP_036212.1	-
Hs.326035	early growth response 1	EGR1	119242	sp:P18146	++
Hs.108885	collagen, type VI, alpha 1	COL6A1	15011913	ref:NP_001839.1	++
Hs.78944	regulator of G-protein signalling 2, 24kDa	RGS2	2135146	pir:I53020	++
Hs.110571	growth arrest and DNA-damage-inducible, beta	GADD45B	9945332	ref:NP_056490.1	++
Hs.78465	v-jun sarcoma virus 17 oncogene homolog (avian)	JUN	135298	sp:P05412	+
Hs.82646	DnaJ (Hsp40) homolog, subfmaily B, member 1	DNAJB1	1706473	sp:P25685	+
Hs.169840	TTK protein kinase	TTK	346403	pir:A42861	+
Hs.211601	mitogen-activated protein kinase kinase kinase 12	MAP3K12	18202489	sp:Q12852	+
Hs.345728	suppressor of cytokine signaling 3	SSI-3	4507235	ref:NP_003946.1	+
Hs.3776	zinc finger protein 216	ZNF216	5174755	ref:NP_005998.1	+
Hs.73037	cannabinoid receptor 2 (macrophage)	CNR2	450068	prf:1920360A	+
Hs.167578	EST,FLJ25357 hypothetical protein FLJ25357		740170	2004399A	+
Hs.8715	hypothetical protein MGC3232	MGC3232	3024681	sp:O00268	+
Hs.74520	spinocerebellar ataxia 1 (olivopontocerebellar ataxia 1, autosomal dominant, ataxin 1)	SCA1	1082237	pir:S46268	+
Hs.6151	pumilio homolog 2 (Drosophila)	PUM2	14277945	pdb:1IB3	+
Hs.8026	EST, Highly similar to SES2_HUMAN Sestrin 2 [H.sapiens]		13633882	sp:P58004	+
Hs.82173	TGFB inducible early growth response	TIEG	11387050	sp:Q13118	+
Hs.198307	von Hippel-Lindau binding protein 1	VBP1	4507873	ref:NP_003363.1	+
Hs.179982	tumor protein p53-binding protein	TP53BPL	5032191	ref:NP_005793.1	+
Hs.2549	adrenergic, beta-3-, receptor	ADRB3	1070630	pir:QRHUBE	+
Hs.2128	dual specificity phosphatase 5	DUSP5	12707566	ref:NP_004410.2	+
Hs.36927	heat shock 105kD	HSP105B	5729879	ref:NP_006635.1	+
Hs.77558	high mobility group nucleosomal binding domain 3	HMGN3	2495254	sp:Q15651	+
Hs.460	activating transcription factor 3	ATF3	88875	pir:C34223	+
Hs.104125	adenylyl cyclase-associated protein	CAP	399184	sp:Q01518	+
Hs.24719	modulator of apoptosis 1	MAP-1	11545896	ref:NP_071434.1	+
Hs.8257	cytokine inducible SH2-containing protein	CISH	13124022	sp:Q9NSE2	+
Hs.101383	ESTs, Weakly similar to A43932 mucin 2 precursor, intestinal - human (fragments)		2135765	pir:A43932	+

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Table 4. Continuation I

Unigene cluster	Description	GENE	gi	pir/NCBI/swisspr	effect
Hs.276770	CDW52 antigen (CAMPATH-1 antigen)	CDW52	4502761	ref:NP_001794.1	+
Hs.8084	hypothetical protein dJ465N24.2.1	DJ465N24.2.1	10092679	ref:NP_064713.1	+
Hs.78829	ubiquitin specific protease 10	USP10	11360280	pir:T47164	+
Hs.889	Charot-Leyden crystal protein	CLC	1942631	pdb:1LCL	+
Hs.277401	bromodomain adjacent to zinc finger domain, 2A	BAZ2A	7304921	ref:NP_038477.1	+
Hs.300863	lethal (3) malignant brain tumor l(3)mbt protein (Drosophila) homolog	H-L(3)MBT	14141728	ref:NP_056293.2	+
Hs.4552	ubiquilin 2	UBQLN2	16753207	ref:NP_038472.2	+
Hs.151903	GrpE-like protein cochaperone	HMGE	18202951	sp:Q9HAV7	+
Hs.36606	EST, Weakly similar to T29982 hypothetical protein F11G11.12 - [C. elegans]				+
Hs.85302	adenosine deaminase, RNA-specific, B1 (RED1 homolog rat)	ADARB1	2829669	sp:P78563	+
Hs.113823	ClpX caseinolytic protease X homolog (E. coli)	CLPX	14916956	sp:O76031	+
Hs.25911	HLA-B associated transcript 2	BAT2	18375626	ref:NP_542417.1	+
Hs.95821	osteoclast stimulating factor 1	OSTF1	11134088	sp:Q92882	+
Hs.11217	KIAA0877 protein	KIAA0877			+
Hs.301064	arfaptin 1	HSU52521	1703203	sp:P53367	+
Hs.276238	EST, Moderately similar to kinase suppressor of ras [Mus musculus]				+
Hs.211569	G protein-coupled receptor kinase 5	GPRK5	2135145	pir:A48277	+
Hs.25524	protein tyrosine phosphatase, non-receptor type 23	PTPN23	7512735	pir:T14756	+
Hs.94498	leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 2	LILRA2	5803068	ref:NP_006857.1	+
Hs.24427	DKFZP566O1646 protein	DC8	7512839	pir:T08737	+
Hs.46	platelet-activating factor receptor	PTAFR	107346	pir:A40191	+
Hs.90800	EST, Highly similar to matrix metalloproteinase 16, isoform 1; membrane-type matrix metalloproteinase 3; membrane-type-3 matrix metalloproteinase [Homo sapiens]		13027802	ref:NP_005932.2	+
Hs.81648	hypothetical protein FLJ11021 similar to splicing factor, arginine/serine-rich 4	FLJ11021	2833266	sp:Q15696	+
Hs.80338	Bcl-2-associated transcription factor	BTF	7661958	ref:NP_055554.1	+
Hs.238407	EST, Weakly similar to hypothetical protein FLJ20489 [Homo sapiens] [H.sapiens]		8923452	ref:NP_060312.1	+
Hs.154668	KIAA0391 gene product	KIAA0391	3024899	sp:O15091	+
Hs.76666	chromosome 9 open reading frame 10	C9orf10	13431358	sp:Q9NZB2	+
Hs.9701	growth arrest and DNA-damage-inducible, gamma	GADD45G	5729836	ref:NP_006696.1	+
Hs.100527	connector enhancer of KSR2	CNK2	7662368	ref:NP_055742.1	+
Hs.77274	plasminogen activator, urokinase	PLAU	224665	prf:1110198A	+

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Table 4. Continuation II

Unigene cluster	Description	GENE	gi	pir/NCBI/swisspr	effect
Hs.93516	ESTs				+
Hs.376709	Homo sapiens cDNA FLJ33768 fis, clone BRHIP2000021				+
Hs.110299	mitogen-activated protein kinase kinase 7	MAP2K7	4826946	ref:NP_005034.1	+
Hs.31396	ESTs, Weakly similar to S28807 collagen alpha 1(X) chain precursor [M.musculus]				+
Hs.129715	gonadotropin-releasing hormone 2	GNRH2	3913735	sp:O43555	+
Hs.169370	FYN oncogene related to SRC, FGR, YES	FYN	125370	sp:P06241	+
Hs.82007	methionyl aminopeptidase 1	METAP1	1703270	sp:P53582	+
Hs.239018	RAB11B, member RAS oncogene family	RAB11B	1082426	pir:JC2487	+
Hs.126852	solute carrier family 6 (neurotransmitter transporter, GABA), member 13	SLC6A13	7705539	ref:NP_057699.1	+

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SEQUENCE LISTING

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<130> (Filename)

<140> (Current Application Number)
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His Ala Asp Gly Ile Cys Arg Asn Arg Arg Gln Cys Asn Arg Glu Val
            20                  25                  30

tgc ggt tct acc tac gat gtg gcc gtc gtg ggg gcg ggg cct ggg gga     144
Cys Gly Ser Thr Tyr Asp Val Ala Val Val Gly Ala Gly Pro Gly Gly
        35                  40                  45

gct aac tcc gcc tac atg ctg agg gac tcc ggc ctg gac atc gct gtg     192
Ala Asn Ser Ala Tyr Met Leu Arg Asp Ser Gly Leu Asp Ile Ala Val
    50                  55                  60

ttc gag tac tcg gac cga gtg ggc ggc cgg ctg ttc acc tac cag ctg     240
Phe Glu Tyr Ser Asp Arg Val Gly Gly Arg Leu Phe Thr Tyr Gln Leu
65                  70                  75                  80

ccc aac aca ccc gac gtt aac ctg gag att ggc ggc atg agg ttc atc     288
Pro Asn Thr Pro Asp Val Asn Leu Glu Ile Gly Gly Met Arg Phe Ile
                85                  90                  95

gaa ggc gcc atg cac agg ctc tgg agg gtc att tca gaa ctc ggc cta     336
Glu Gly Ala Met His Arg Leu Trp Arg Val Ile Ser Glu Leu Gly Leu
            100                 105                 110

acc ccc aag gtg ttc aag gaa ggt ttc ggc aag gag ggc aga caa aga     384
Thr Pro Lys Val Phe Lys Glu Gly Phe Gly Lys Glu Gly Arg Gln Arg
        115                 120                 125

ttt tac ctg cgg gga cag agc ctg acc aag aaa cag gtc aag agt ggg     432
Phe Tyr Leu Arg Gly Gln Ser Leu Thr Lys Lys Gln Val Lys Ser Gly
    130                 135                 140

gac gta ccc tat gac ctc agc ccg gag gag aaa gaa aac cag gga aat     480
Asp Val Pro Tyr Asp Leu Ser Pro Glu Glu Lys Glu Asn Gln Gly Asn
145                 150                 155                 160

ctg gtc gaa tac tac ctg gag aaa ctg aca ggt cta caa ctc aac ggc     528
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caa aga gtc atc ctg gcc atg ccg gta tac gct ctg aac caa cta gac 1008
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Trp Asn Gln Leu Arg Asn Asp Arg Ala Thr Gln Ala Tyr Ala Ala Val
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Arg Pro Ile Pro Ala Ser Lys Val Phe Met Ser Phe Asp Gln Pro Trp
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aac cag gtt acc gaa ccc ctc aag gac acc att ctt gac cac ctc act 1344
Asn Gln Val Thr Glu Pro Leu Lys Asp Thr Ile Leu Asp His Leu Thr
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gct tcc cag ttc tgg aca gac tac ccg ttt ggc tgt gga tgg atc acc 1440
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465 475 480

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Glu Pro Leu Lys Arg Glu Val Ala Leu Lys Leu Thr Val Pro Asp Gly
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Glu Val Thr Leu Asp Ala Ser Ala Val Ser Leu Phe Asp Asp His Leu
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Gly Ser Thr Tyr Asp Val Ala Val Val Gly Ala Gly Pro Gly Gly Ala
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Asn Ser Ala Tyr Met Leu Arg Asp Ser Gly Leu Asp Ile Ala Val Phe
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gag tac tca gac cga gtg ggc ggc cgg ctg ttc acc tac cag ctg ccc 240
Glu Tyr Ser Asp Arg Val Gly Gly Arg Leu Phe Thr Tyr Gln Leu Pro
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ccc aag gtg ttc aag gaa ggt ttc gga aag gag ggc aga cag aga ttt      384
Pro Lys Val Phe Lys Glu Gly Phe Gly Lys Glu Gly Arg Gln Arg Phe
        115                 120                 125

tac ctg cgg gga cag agc ctg acc aag aaa cag gtc aag agt ggg gac      432
Tyr Leu Arg Gly Gln Ser Leu Thr Lys Lys Gln Val Lys Ser Gly Asp
    130                 135                 140

gta ccc tat gac ctc agc ccg gag gag aaa gaa aac cag gga aat ctg      480
Val Pro Tyr Asp Leu Ser Pro Glu Glu Lys Glu Asn Gln Gly Asn Leu
145                 150                 155                 160

gtc gaa tac tac ctg gag aaa ctg aca ggt cta caa ctc aat ggt gaa      528
Val Glu Tyr Tyr Leu Glu Lys Leu Thr Gly Leu Gln Leu Asn Gly Glu
                165                 170                 175

ccg ctc aaa cgt gag gtt gcg ctt aaa cta acc gtg ccg gac ggc aga      576
Pro Leu Lys Arg Glu Val Ala Leu Lys Leu Thr Val Pro Asp Gly Arg
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ttc ctc tat gac ctc tcg ttt gac gaa gcc atg gat ctg gtt gcc tcc      624
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cct gag ggc aaa gag ttc acc cga gac acg cac gtc ttc acc gga gag      672
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gtc acc ctg ggc gcg tcg gct gtc tcc ctc ttc gac gac cac ctg gga      720
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aga gtc att ctg gcc atg ccg gtc tac gct ctc aac cag ttg gat tgg     1008
Arg Val Ile Leu Ala Met Pro Val Tyr Ala Leu Asn Gln Leu Asp Trp
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ctg aag aat cag gga gag gac atc cca ggc tct gac cca ggc tac aac 1296
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Arg Val Ile Leu Ala Met Pro Val Tyr Ala Leu Asn Gln Leu Asp Trp
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Asn Gln Leu Arg Asn Asp Arg Ala Thr Gln Ala Tyr Ala Ala Val Arg
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355 360 365

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Ser Gln Met Tyr Asp Trp Gln Lys Ser Glu Ala Ser Gly Asp Tyr Ile
385 390 395 400

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405 410 415

Leu Lys Asn Gln Gly Glu Asp Ile Pro Gly Ser Asp Pro Gly Tyr Asn
420 425 430

Gln Val Thr Glu Pro Leu Lys Asp Thr Ile Leu Asp His Leu Thr Glu
435 440 445

Ala Tyr Gly Val Glu Arg Asp Ser Ile Arg Glu Pro Val Thr Ala Ala
450 455 460

Ser Gln Phe Trp Thr Asp Tyr Pro Phe Gly Cys Gly Trp Ile Thr Trp
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Arg Ala Gly Phe His Phe Asp Asp Val Ile Ser Thr Met Arg Arg Pro
485 490 495

Ser Leu Lys Asp Glu Val Tyr Val Val Gly Ala Asp Tyr Ser Trp Gly
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Leu Ile Ser Ser Trp Ile Glu Gly Ala Leu Glu Thr Ser Glu Asn Val
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Ile Asn Asp Tyr Phe Leu
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Ser Thr Tyr Asp Val Ala Val Val Gly Ala Gly Pro Gly Gly Ala Asn
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Tyr Ser Asp Arg Val Gly Gly Arg Leu Phe Thr Tyr Gln Leu Pro Asn
    50                  55                  60

aca ccc gac gtt aat ctc gag att ggc ggc atg agg ttc atc gag ggc        240
Thr Pro Asp Val Asn Leu Glu Ile Gly Gly Met Arg Phe Ile Glu Gly
65                  70                  75                  80

gcc atg cac agg ctc tgg agg gtc att tca gaa ctc ggc cta acc ccc        288
Ala Met His Arg Leu Trp Arg Val Ile Ser Glu Leu Gly Leu Thr Pro
                85                  90                  95

aag gtg ttc aag gaa ggt ttc gga aag gag ggc aga cag aga ttt tac        336
Lys Val Phe Lys Glu Gly Phe Gly Lys Glu Gly Arg Gln Arg Phe Tyr
            100                 105                 110

ctg cgg gga cag agc ctg acc aag aaa cag gtc aag agt ggg gac gta        384
Leu Arg Gly Gln Ser Leu Thr Lys Lys Gln Val Lys Ser Gly Asp Val
        115                 120                 125

ccc tat gac ctc agc ccg gag gag aaa gaa aac cag gga aat ctg gtc        432
Pro Tyr Asp Leu Ser Pro Glu Glu Lys Glu Asn Gln Gly Asn Leu Val
    130                 135                 140

gaa tac tac ctg gag aaa ctg aca ggt cta aaa ctc aac ggc gga ccg        480
Glu Tyr Tyr Leu Glu Lys Leu Thr Gly Leu Lys Leu Asn Gly Gly Pro
145                 150                 155                 160

ctc aaa cgt gag gtt gcg ctt aaa cta acc gtg ccg gac ggc aga ttc        528
Leu Lys Arg Glu Val Ala Leu Lys Leu Thr Val Pro Asp Gly Arg Phe
                165                 170                 175

ctc tat gac ctc tcg ttt gac gaa gcc atg gac ctg gtt gcc tcc cct        576
Leu Tyr Asp Leu Ser Phe Asp Glu Ala Met Asp Leu Val Ala Ser Pro
            180                 185                 190

gag ggc aaa gag ttc acc cga gac acg cac gtg ttc acc gga gaa gtc        624
Glu Gly Lys Glu Phe Thr Arg Asp Thr His Val Phe Thr Gly Glu Val
        195                 200                 205

acc ctg gac gcg tcg gct gtc tcc ctc ttc gac gac cac ctg gga gag        672
Thr Leu Asp Ala Ser Ala Val Ser Leu Phe Asp Asp His Leu Gly Glu
    210                 215                 220

gac tac tat ggc agt gag atc tac acc cta aag gaa gga ctg tct tcc        720
Asp Tyr Tyr Gly Ser Glu Ile Tyr Thr Leu Lys Glu Gly Leu Ser Ser
225                 230                 235                 240

gtc cca caa ggg ctc cta cag act ttt ctg gac gcc gca gac tcc aac        768
Val Pro Gln Gly Leu Leu Gln Thr Phe Leu Asp Ala Ala Asp Ser Asn
                245                 250                 255

gag ttc tat ccc aac agc cac ctg aag gcc ctg aga cgt aag acc aac        816
Glu Phe Tyr Pro Asn Ser His Leu Lys Ala Leu Arg Arg Lys Thr Asn
            260                 265                 270

ggt cag tat gtt ctt tac ttt gag ccc acc acc tcc aag gat gga caa        864
Gly Gln Tyr Val Leu Tyr Phe Glu Pro Thr Thr Ser Lys Asp Gly Gln
        275                 280                 285

acc aca atc aac tat ctg gaa ccc ctg cag gtt gtg tgt gca cag aga        912
Thr Thr Ile Asn Tyr Leu Glu Pro Leu Gln Val Val Cys Ala Gln Arg
    290                 295                 300

gtc atc ctg gcc atg ccg gtc tac gct ctc aac caa ctg gac tgg aat        960
Val Ile Leu Ala Met Pro Val Tyr Ala Leu Asn Gln Leu Asp Trp Asn
305                 310                 315                 320
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cag ctc aga aat gac [illegible] gcc acc caa gcg tac gct gcc gtg c[illegible] ccg     1008
Gln Leu Arg Asn Asp [illegible] Ala Thr Gln Ala Tyr Ala Ala Val Arg Pro
                325                     330                     335

att cct gca agt aaa gtg ttc atg acc ttt gat cag ccc tgg tgg ttg     1056
Ile Pro Ala Ser Lys Val Phe Met Thr Phe Asp Gln Pro Trp Trp Leu
            340                 345                 350

gag aac gag agg aaa tcc tgg gtc acc aag tcg gac gcg ctt ttc agc     1104
Glu Asn Glu Arg Lys Ser Trp Val Thr Lys Ser Asp Ala Leu Phe Ser
        355                 360                 365

caa atg tac gac tgg cag aag tct gag gcg tcc gga gac tac atc ctg     1152
Gln Met Tyr Asp Trp Gln Lys Ser Glu Ala Ser Gly Asp Tyr Ile Leu
    370                 375                 380

atc gcc agc tac gcc gac ggc ctc aaa gcc cag tac ctg cgg gag ctg     1200
Ile Ala Ser Tyr Ala Asp Gly Leu Lys Ala Gln Tyr Leu Arg Glu Leu
385                 390                 395                 400

aag aat cag gga gag gac atc cca ggc tct gac cca ggc tac aac cag     1248
Lys Asn Gln Gly Glu Asp Ile Pro Gly Ser Asp Pro Gly Tyr Asn Gln
                405                 410                 415

gtc acc gaa ccc ctc aag gac acc att ctt gac cac ctc act gag gct     1296
Val Thr Glu Pro Leu Lys Asp Thr Ile Leu Asp His Leu Thr Glu Ala
            420                 425                 430

tat ggc gtg gaa cga gac tcg atc ccg gaa ccc gtg acc gcc gct tcc     1344
Tyr Gly Val Glu Arg Asp Ser Ile Pro Glu Pro Val Thr Ala Ala Ser
        435                 440                 445

cag ttc tgg acc gac tac ccg ttc ggc tgt gga tgg atc acc tgg agg     1392
Gln Phe Trp Thr Asp Tyr Pro Phe Gly Cys Gly Trp Ile Thr Trp Arg
    450                 455                 460

gca ggc ttc cat ttt gat gac gtc atc agc acc atg cgt cgc ccg tca     1440
Ala Gly Phe His Phe Asp Asp Val Ile Ser Thr Met Arg Arg Pro Ser
465                 470                 475                 480

ctg aaa gat gag gtc tac gtg gtg gga gcc gat tac tcc tgg gga ctt     1488
Leu Lys Asp Glu Val Tyr Val Val Gly Ala Asp Tyr Ser Trp Gly Leu
                485                 490                 495

atc tcc tcc tgg ata gag ggc gct ctg gag acc tcg gaa aac gtc atc     1536
Ile Ser Ser Trp Ile Glu Gly Ala Leu Glu Thr Ser Glu Asn Val Ile
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aac gac tac ttc ctc taa                                             1554
Asn Asp Tyr Phe Leu
        515
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<211> 517

<212> PRT

<213> Aplysia punctata

<400> 6

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Ser Thr Tyr Asp Val Ala Val Val Gly Ala Gly Pro Gly Gly Ala Asn
            20                  25                  30
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Ser Ala Tyr Met Leu A[illegible]sp Ser Gly Leu Asp Ile Ala Val P[illegible]lu
        35                  40                  45

Tyr Ser Asp Arg Val Gly Gly Arg Leu Phe Thr Tyr Gln Leu Pro Asn
    50                  55                  60

Thr Pro Asp Val Asn Leu Glu Ile Gly Gly Met Arg Phe Ile Glu Gly
65                  70                  75                  80

Ala Met His Arg Leu Trp Arg Val Ile Ser Glu Leu Gly Leu Thr Pro
                85                  90                  95

Lys Val Phe Lys Glu Gly Phe Gly Lys Glu Gly Arg Gln Arg Phe Tyr
            100                 105                 110

Leu Arg Gly Gln Ser Leu Thr Lys Lys Gln Val Lys Ser Gly Asp Val
        115                 120                 125

Pro Tyr Asp Leu Ser Pro Glu Glu Lys Glu Asn Gln Gly Asn Leu Val
    130                 135                 140

Glu Tyr Tyr Leu Glu Lys Leu Thr Gly Leu Lys Leu Asn Gly Gly Pro
145                 150                 155                 160

Leu Lys Arg Glu Val Ala Leu Lys Leu Thr Val Pro Asp Gly Arg Phe
                165                 170                 175

Leu Tyr Asp Leu Ser Phe Asp Glu Ala Met Asp Leu Val Ala Ser Pro
            180                 185                 190

Glu Gly Lys Glu Phe Thr Arg Asp Thr His Val Phe Thr Gly Glu Val
        195                 200                 205

Thr Leu Asp Ala Ser Ala Val Ser Leu Phe Asp Asp His Leu Gly Glu
    210                 215                 220

Asp Tyr Tyr Gly Ser Glu Ile Tyr Thr Leu Lys Glu Gly Leu Ser Ser
225                 230                 235                 240

Val Pro Gln Gly Leu Leu Gln Thr Phe Leu Asp Ala Ala Asp Ser Asn
                245                 250                 255

Glu Phe Tyr Pro Asn Ser His Leu Lys Ala Leu Arg Arg Lys Thr Asn
            260                 265                 270

Gly Gln Tyr Val Leu Tyr Phe Glu Pro Thr Thr Ser Lys Asp Gly Gln
        275                 280                 285

Thr Thr Ile Asn Tyr Leu Glu Pro Leu Gln Val Val Cys Ala Gln Arg
    290                 295                 300

Val Ile Leu Ala Met Pro Val Tyr Ala Leu Asn Gln Leu Asp Trp Asn
305                 310                 315                 320

Gln Leu Arg Asn Asp Arg Ala Thr Gln Ala Tyr Ala Ala Val Arg Pro
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325 330 3

Ile Pro Ala Ser Lys Val Phe Met Thr Phe Asp Gln Pro Trp Trp Leu
340 345 350

Glu Asn Glu Arg Lys Ser Trp Val Thr Lys Ser Asp Ala Leu Phe Ser
355 360 365

Gln Met Tyr Asp Trp Gln Lys Ser Glu Ala Ser Gly Asp Tyr Ile Leu
370 375 380

Ile Ala Ser Tyr Ala Asp Gly Leu Lys Ala Gln Tyr Leu Arg Glu Leu
385 390 395 400

Lys Asn Gln Gly Glu Asp Ile Pro Gly Ser Asp Pro Gly Tyr Asn Gln
405 410 415

Val Thr Glu Pro Leu Lys Asp Thr Ile Leu Asp His Leu Thr Glu Ala
420 425 430

Tyr Gly Val Glu Arg Asp Ser Ile Pro Glu Pro Val Thr Ala Ala Ser
435 440 445

Gln Phe Trp Thr Asp Tyr Pro Phe Gly Cys Gly Trp Ile Thr Trp Arg
450 455 460

Ala Gly Phe His Phe Asp Asp Val Ile Ser Thr Met Arg Arg Pro Ser
465 470 475 480

Leu Lys Asp Glu Val Tyr Val Val Gly Ala Asp Tyr Ser Trp Gly Leu
485 490 495

Ile Ser Ser Trp Ile Glu Gly Ala Leu Glu Thr Ser Glu Asn Val Ile
500 505 510

Asn Asp Tyr Phe Leu
515